

# **Ion Metabolism in Halophilic Organisms**

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## FOREWORD

This is one of a continuing series of reports designed to present accounts of progress in saline water conversion and the economics of its application. Such data are expected to contribute to the long-range development of economical processes applicable to low-cost demineralization of sea and other saline water.

Except for minor editing, the data herein are as contained in a report submitted by the contractor. The data and conclusions given in the report are essentially those of the contractor and are not necessarily endorsed by the Department of the Interior.

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## ABSTRACT

Work is described on 2 species of unicellular organisms isolated from the Dead Sea. The first is a species of Halobacterium requiring 3.5 Molar NaCl (20% salt solution) for optimum growth. The second is a green alga and grows best in 1.0-1.5 Molar NaCl (6-10% salt solution) although it can grow at salt concentrations approaching saturation. In the Dead Sea the bacteria live on organic matter swept in from the inflowing streams. Both organisms live in surface waters, the bacteria because they need oxygen and the algae because they need light.

For a long time it has been considered a mystery as to how living organisms manage to tolerate salt concentrations as high as those found in the Dead Sea. It was thought that their mechanisms for regulating the salts of the cell interior must be very powerful and that a study of these mechanisms might advance our knowledge of biological desalination. The study described here has justified itself in that it had demonstrated a completely new and highly powerful system for salt-regulation, capable of controlling the concentration of sodium chloride from 0.4 to 4 Molar (2.4% - 24% salt). The system found in Halobacterium has not so far been described anywhere else in the plant or animal kingdom.

Both the Halobacterium species and D. parva have been found to be highly permeable to the hydrogen ion and to the ions of the alkali metals. Halobacterium cells are even permeable to large organic molecules including starch. Thus the cells of these organisms are not surrounded by any functional membrane and differ in this respect from the cells of other known species.

The ion metabolism of the Halobacterium species has been studied in some detail. It was already known to contain large amounts of potassium; this ion has been found at high, and fairly even concentrations within the bacteria at all stages of their life-cycle although the outside concentration is very low. Sodium, on the other hand, was found in high concentrations in young, active cultures and in low concentrations in old cultures despite the high outside concentration. Now this is the opposite situation to that found in other bacteria, in which old sluggish cultures are marked by little or no ion gradients across the cell membrane. The finding in Halobacterium showed that the forces excluding sodium from the cell interior were not due to metabolic energy. This finding was confirmed in a number of other ways, for example by cooling the bacteria, and by starving them, and generally by making it absolutely clear that the means possessed by the bacteria for controlling their sodium concentration did not depend on the expenditure of metabolic energy. Since every other cell system studied up to now is apparently dependent on a continuous output of energy for the regulation of cell ions, it means that the Halobacterium species studied here is a unique system.

It appears that the mechanism for salt control in Halobacterium lies in the structure of the cell proteins and in the form taken by potassium within the cell. Changes in either of these 2 parameters lead to changes in the concentration of cell sodium. Thus the site of control is within the cell interior, rather than in the cell membrane, as is the case in other organisms.

Membrane permeability of Halobacterium sp.  
and Dunaliella parva.

ABSTRACT

The permeability of a Halobacterium species and Dunaliella parva to several radioactive substances has been measured. The method used was to centrifuge a cell suspension, within which was dissolved the given radioactive substance, and measure the radioactivity of the resultant cell pellet and supernatant. The volume of radioactive substance within the cell pellet was then calculated. Both organisms were found to be impermeable to ( $^{14}\text{C}$ ) dextran (mol. wt. 80,000). Results indicated that the Halobacterium cell was freely permeable to ( $^{14}\text{C}$ )-sucrose, ( $^{14}\text{C}$ ) inulin, and even ( $^{14}\text{C}$ ) polyvinylpyrrolidone (mol. wt. 30,000-40,000). A region of the D. parva cell was permeable to ( $^{14}\text{C}$ ) sucrose and to ( $^{14}\text{C}$ ) inulin. It is argued that the results indicate the existence of large pores in the cell membranes of these two organisms.

Ginzburg, M. 1969. The unusual membrane permeability of two halophilic unicellular organisms. Biochim. Biophys. Acta, 173:370-376.

The high permeability of the D. parva membrane has been exploited to study an inrush of hydrogen ions brought about by sudden illumination. Although this inrush has been seen before in isolated chloroplasts it has not before been studied in whole cells. In the cells of D. parva the hydrogen-ion inrush is 10 times greater than in isolated chloroplasts; its importance lies in its connection with the synthesis of high-energy compounds used to drive the cell's metabolism.

The 2 halophilic organisms in question have opened new fields of study and have thrown totally unexpected light on processes formerly thought to be understood.

Effect of age of culture on ion concentrations  
of Halobacterium sp.

## INTRODUCTION

It has been known for some time that halophilic bacteria, living in an environment containing 2M NaCl or more, themselves contain large amounts of salt, including 3M potassium/liter cell water (1). It is very puzzling to know how any organism can tolerate such high internal salt concentrations and how the mechanisms for ion transport can operate under such conditions of high external salinity. Thus an investigation of ion transport in halophilic bacteria is certain to be rewarding. It could not be undertaken, however, until methods had been developed for the study of ion concentrations in single-celled cultures and in small unicellular organisms. Such methods have been developed recently and have been applied in particular to Escherichia coli and yeast. Work on E. coli has been published in a series of papers in the Journal of General Physiology starting in 1961 (2). Rothstein (3) has summarized work on yeast up to 1964. Other unicellular systems studied include Streptococcus fecalis (4). Chlorella (5) and the Hela cell (6).

Up to the present, methods for studying ion transport in cell suspensions have depended on the isolation of uniform pellets centrifuged from concentrated cell suspensions or on the filtration, followed by washing, of undisturbed cell suspensions. An important feature of the work to be described here is that ion concentrations have been measured in dilute suspensions of bacteria, by separation of the bacteria from the medium without any washing procedure. Such a technique was necessary since halophilic bacteria have no rigid cell-wall (7) and are extremely sensitive to usual experimental procedures (e.g. pipetting, washing with sucrose solutions) which cause cell breakage and loss of a portion of the cell population (7, 8).

It is the purpose of this paper to show how intracellular K, Na and Cl concentrations in a strain of halophilic bacteria change during the course of growth.

## ISOLATION AND IDENTIFICATION OF ORGANISM

A sample of water from the Dead Sea was centrifuged and the residue streaked onto an agar plate containing the substance listed in Table 1, with the addition of 5% Bacto-tryptone. After several days round pinkish colonies were observed. One of these was restreaked onto agar and then inoculated into a liquid medium of the same composition. The resulting bacterial culture was found to grow with a generation time of 5-6 hours.

Preliminary trials established that the optimum concentration of NaCl for growth was 3.5 - 4.0 M. 3.5 Molar NaCl was chosen as a working concentration. No growth was observed at concentrations lower than 2.0 M NaCl.

The bacteria were highly pleomorphic but showed a tendency towards the rod shape. The color of suspensions was pink, the color deepening as the cultures aged. These 2 characteristics together with the obligately halophilic nature of the bacteria, show them to belong to the genus *Halobacterium* (Bergey's manual of determinative bacteriology, 7th edition).

The species has not yet been determined.

### CHEMICALS

All salts used were of Analytical Reagent grade. The de-ionized water used throughout the course of this investigation had a conductivity of less than 1 Mho / cm.

### METHODS OF CULTURE

#### Medium

The composition of the medium of growth is shown in Table 1. It was prepared by dissolving all the salts except Mg in water. Yeast autolysate was

added and the pH adjusted to 0.3 pH units above the value desired for the final medium. The addition of  $\text{MgSO}_4$  at this point lowered the pH to the desired final value.  $\text{MgSO}_4$  was added last because any alkalisation of the medium in its presence caused precipitation of Mg compounds.

The medium was sterilised by filtration through autoclaved Millipore filters HAWP (pore diameter 0.45  $\mu$ ).

Yeast autolysate was prepared by heating equal weights of baker's yeast and water at 80°C for 10 minutes. The cooled mixture was centrifuged and the supernatant filtered through Millipore filters until the solution was clear. Only 2 weeks' supply was prepared at any one time, since the autolysate was found to deteriorate with time.

The yeast autolysate formed the sole source of carbon and nitrogen.

#### Methods of Culture

150 mls. medium were poured in a sterile manner into 500 ml. Erlenmeyer flasks with an inserted side-arm, consisting of a Klett tube, to permit measurement of the optical density of the growing culture. The flasks were incubated in a water-bath at 37°C on a horizontal shaker. Growth was measured by means of a Klett photometer (blue filter, 400-465 m $\mu$ ).

#### Maintenance of Bacterial Stocks

Stocks were carried on agar slants consisting of growth medium solidified with 1.5% agar. The stocks were transferred every 2 months onto fresh slants. A new stock was taken for experiments every 2 weeks. Periodically, the purity of the culture was checked by plating out very dilute suspensions.

## RESUSPENSION OF BACTERIA

When it was necessary to resuspend the bacteria, they were centrifuged at 5900 g in a Sorvall refrigerated centrifuge (model RC2 - B) at 20°C, for 10 minutes. The button of red bacterial mass, which had collected at the base of the tube, was transferred with a spatula to the required volume of fresh medium. Rapid stirring of the medium by means of a magnetic bar, brought about resuspension of the bacteria within 20 minutes. On resuspension, such cells resumed growth, as measured by protein synthesis, at their former rate.

Lysed cells did not sediment during centrifugation.

Resuspension and concentration of bacterial suspensions was required for measurement of the dry weight content and trapped volume. The bacteria were not resuspended for any other measurement described in this paper.

Resuspension brought about swelling of the bacterial cells. The degree of swelling could be measured by comparing cell protein or K content with cell volume before and after resuspension.

## MEASUREMENTS OF CELL MASS

The following criteria were used as measurements of bacterial mass:

a) protein, b) volume of pellet after centrifugation under standard conditions, c) % dry weight. Protein and pellet volumes were measured in the course of each experiment. The % dry weight was determined at various stages of the growth cycle of the culture. Measurements b) and c) required correction for supernatant trapped within the pellet during centrifugation, in order for the volume and % dry weight of the cell material to be calculated.

a) Protein

250  $\mu$ l samples of suspension were centrifuged in polyethylene tubes in a Beckman Microfuge for 5 minutes. The tip of the tube containing the bacteria

was cut off with a clean razor blade and dropped into a glass test-tube to which was added 0.5 mls 1N NaOH. The protein was determined according to Lowry's method (9), using bovine serum albumin as standard. Protein determinations on whole cells extracted with NaOH and on trichloro acetic acid precipitates of whole cells agreed to within 1%. Thus, routinely, whole cells extracted with NaOH were used. As a check on the Lowry method, the same trichloro acetic acid precipitate was used both for a set of Lowry determinations and for measurement of nitrogen, assuming that nitrogen was 14.7% by weight of total protein. In 2 separate determinations the protein, as measured by the Lowry method, was  $81.5 \pm 1.5\%$  of the amount of protein calculated from the measurement from total N. The protein results quoted throughout this paper, are those obtained by use of the Lowry method. In any one determination the protein samples were measured in triplicate. Agreement between replicates was to within 3%.

b) Volume

The volume of bacterial pellets was determined by centrifuging bacterial suspensions in cytocrit tubes consisting of a glass bulb of 3-4 mls. capacity connected to a precision-bore capillary tube of 1 mm. diameter (Chance Bros., Smethwick, England). The tube was sealed at the base; to overcome the deformity due to the sealing, at the extreme base of the tube, a drop of mercury was introduced into the base.

Three mls. of bacterial suspension were introduced into the tube by means of a syringe. The tubes were centrifuged at 13,000 g for 30 minutes. The length of the resultant cell column was measured by means of an ocular scale in the eyepiece of a binocular microscope. The volume of the pellet was calculated from its length, the diameter of the tube being known.

The horizontal centrifuge was designed and built by Mr. E. Sochatchewer.

c) Fresh Weight and Dry Weight of Bacterial Pellets

1) By extrusion of pellet onto disc.

This method was first described by Schultz and Solomon (2). A bacterial suspension (not less than 2% of total volume occupied by cells) is centrifuged in cytocrit tubes similar to those described in the previous section, but with the bases closed by teflon plugs. After centrifugation, portions of the resultant cell columns are extruded onto tared aluminium discs and weighed on a Misco quartz helix balance. The weighed pellets are dried at 90°C to constant weight and reweighed. The ratio of the dry weight to total fresh weight can then be calculated.

Several determinations from bacteria in the early logarithmic phase were made by this method. (See Table 2).

2) By measurement of pellet within cytocrit tube

This method has been described by Bentzel and Solomon (10). The bacterial suspension (about 2% cell per total volume) is centrifuged in a tared tube of the same shape as described above. After centrifugation, the supernatant is drawn out by means of a syringe, and the portion of the tube above the cells is rinsed with water and dried with acetone. The tube is then weighed, to obtain the fresh weight of the pellet, and dried for 48 hours at 90°C. It is reweighed to obtain the dry weight of the pellet.

Measurement of supernatant trapped in centrifuged cytocrit pellets

The methods whereby were measured the volume of supernatant trapped within bacterial pellets after centrifugation of suspensions in cytocrit tubes, have been described elsewhere (11). The volume of medium trapped after 30 minutes centrifugation at 13,000 g was  $21.1\% \pm 0.5\%$  (S. E.) of the total pellet volume (mean of 14 determinations). The trapped volume was measured with dextran-<sup>14</sup>C.

## ION MEASUREMENTS

At the beginning of the investigation ion measurements were made on pellets extruded from cytocrit tubes. Owing to the lengthy period of centrifugation needed and the difficulties involved in extruding the poorly-packed pellets, the method was abandoned and use was made of the Beckman Microfuge. This enabled ion determinations to be made on small (250  $\mu$ l) samples of dilute cell suspensions which could be tested rapidly, since the period of centrifugation is 5 minutes or less.

### Total Potassium

250  $\mu$ l of bacterial suspension were introduced into 400  $\mu$ l polyethylene tubes and centrifuged in a Beckman Microfuge for 5 minutes. The tip containing the cells was cut off with a clean razor blade and transferred to a Vycor quartz glass tube. 5 mls. de-ionized water were added and the tube shaken on a Vortex mixer until the cells (which are red in color) were seen to leave the cut tip. A clear liquid was obtained.

First, the total Na was measured with a flame photometer (Eppendorf Model 700) using a propane-butane flame.

K standards containing the concentrations of Na measured in the samples were prepared. This precaution is necessary since high Na concentrations interfere with K determinations made on this flame photometer. The K concentration were measured, and the amount of K present in each sample calculated.

Samples for total K determination were taken in triplicate, and agreed to  $\pm 0.7\%$  of the total K.

### Sodium and Chloride

For the measurement of these ions, samples of bacterial suspension were introduced into Microfuge tubes containing a 50  $\mu$ l drop of dimethyl phthalate (sp. gr. 1.191). On centrifugation, the cells, accompanied by 25.6% of their volume

of supernatant, sedimented below the phthalate which is of lower specific gravity. The measurement of amount of supernatant trapped within the cell pellet is described elsewhere (11). The supernatant (apart from the amount accompanying the cells) stayed above the ester. This technique was first used by Ballentine and Burford (12) for red blood cells.

The tube tip containing the bacteria was rinsed in distilled water and cut with a new razor blade, precautions being taken to avoid contamination. The tip was dropped into a Vycor quartz glass tube and the cells dispersed in 5 mls. distilled water.

The K and Na concentrations of the solution thus obtained were measured with the flame photometer. The ratio of the Na : K concentrations was then calculated.

3 mls. of the same solution were removed to a separate vial for measurement of Cl. This ion was measured by means of a Buchler-Cotlove chloridometer. An additional 1 ml. portion was heated to dryness, ashed with 200  $\mu$ l concentrated  $\text{HNO}_3$ , and the K concentration of the dissolved ash measured. The ratio of the Cl : K concentrations in the original solution was calculated.

Samples were taken in quadruplicate; the standard deviations were  $\pm 6\%$  of the mean for Na : K and  $\pm 5\%$  of the mean for Cl : K.

The ashing step was needed to eliminate the dimethyl phthalate which tended to reduce flame photometer readings without affecting chloridometer readings. In fact, the dimethyl phthalate affected readings only in samples with low K concentrations (10  $\mu$ Eq/ l. or less), or when the solutions had been shaken particularly vigorously for extraction of the cell pellet from the tube tip.

Even after ashing, K as measured after centrifugation of cells with dimethyl-phthalate was lower than that measured without the phthalate. This is because the cells of lowest density did not centrifuge through the phthalate, and hence were not

included in the cell pellet. K, as measured after ashing, was 80-90% of the K measured in absence of dimethyl phthalate.

### Checks on Methods of Extraction

#### a) In absence of dimethyl phthalate

A series of cell pellets was prepared in the usual way and dispersed in distilled water. 1 ml. -portions of the resultant solutions were heated to dryness and ashed with 200  $\mu$ l HNO<sub>3</sub> at 140°C. The ash was dissolved in 1 ml. distilled water. The K concentrations were read in the solutions obtained before and after ashing and were found to agree to within 1.6% (mean of 5 samples). It was concluded that water-extraction of the pellets liberated all the cell K.

#### b) In presence of dimethyl phthalate

The above procedure was repeated on pellets obtained by centrifugation of samples of bacterial suspension with dimethyl phthalate. The Na / K ratios were as follows:

	<u>Water-extraction</u>	<u>After ashing</u>
Experiment (1)	0.73 $\pm$ 0.02 (mean of 4 replicates)	0.77 $\pm$ 0.06 (mean of 4 replicates)
Experiment (2)	0.93 $\pm$ 0.08 (mean of 5 replicates)	0.94 $\pm$ 0.08 (mean of 5 replicates)

It was again concluded that it sufficed to extract the pellets with water in order to extract all the K and Na present.

#### c) For chloride

Chloride was checked according to the method of Cotlove (13) who mentions that protein and peroxide are the major substances interfering with determination of chloride. Protein was destroyed by boiling for 30 minutes with 0.6N NaOH and then centrifuging off the precipitate obtained after addition of acid zinc sulphate.

In samples in which the protein had been hydrolysed in this way, the chloride content was 104.6% of the water-extracted samples. Peroxides were destroyed by treatment with sodium perborate. In such samples, the chloride content was 98.0% of that measured in the water-extracted samples. It was concluded that extraction of samples with water was sufficient.

#### CALCULATIONS OF FRESH AND DRY WEIGHTS OF CELLS

This is calculated by correcting the measured dry/fresh weights of bacterial pellets for the trapped volume of supernatant:

$$\frac{A}{B} = \frac{(D_p) (V_p) (P_p)/100 - (V_s) (D_s)}{(V_p) (P_p) - (V_s) (P_s)}$$

A/B = Dry/Fresh weights of cells

D<sub>p</sub> = measured dry : fresh weights of pellet, per 100 mg fresh weight of pellet

V<sub>p</sub> = Volume of fresh pellet, i. e. 100

P<sub>p</sub> = Density of fresh pellet, i. e. 1.18, obtained from measured ratios of weight and volumes of pellets before drying.

(V<sub>p</sub>) (P<sub>p</sub>) = Fresh weight of pellet

V<sub>s</sub> = Trapped volume of supernatant, i. e. 32.1% of total volume

D<sub>s</sub> = Weight of dried residue of supernatant, i. e. 0.24 mg/ml. as measured on 1 ml. samples of supernatant heated to dryness

P<sub>s</sub> = Density of supernatant i. e. 1.153, as measured on 1 ml. samples of supernatant.

(V<sub>s</sub>) (P<sub>s</sub>) = Weight of trapped supernatant.

The dry : fresh weight ratio thus obtained is that of the cells of the concentrated cell suspension on which measurements were made. In order to

know the dry : fresh weight ratio of cells of the original suspension before it was concentrated by centrifugation, the ratio must be corrected for the cell swelling which occurs during centrifugation. This swelling amounted to  $13 \pm 1\%$  of the original volume (mean of 5 experiments made at different phases of the growth cycle). Values corrected for swelling are given in the final column of Table 2.

### CALCULATIONS INVOLVING IONS

The errors involved in the calculation of amounts and concentrations of ions have been calculated according to Wilson (14):

$$\sigma^2 = \left( \frac{\partial F}{\partial x_i} \right)^2 \sigma_i^2$$

$\sigma^2$  = variance of final result

F = known functional form

$x_i$  = component i

$\sigma_i^2$  = variance of ith component

#### Total K, Na, Cl

The amount of total K can be calculated directly from the K concentration measured with the flame photometer. A small correction must be made for the amount of K present in the supernatant above the cells but cut off with the tip of the tube, and which is estimated from the Na concentration measured at the same time (see below).

Total Na and Cl are calculated by multiplying the Na/K and Cl/K ratios by the total K.

### A. Total K

Volume of bacterial suspension centrifuged in microfuge tube: 250  $\mu$ l.

Cell pellet dispersed in 5 mls. distilled water.

Na concentration : 1.5 mM/1 . . . Amount of Na = 7.5  $\mu$ Eq

K concentration : 202  $\pm$  4  $\mu$ Eq/1 . . . Amount of K = 1.01  $\pm$  0.02  $\mu$ Eq

Supernatant Na concentration = 3.5 Moles per liter  
= 3.5  $\mu$ Eq per  $\mu$ l

On the assumption that all the measured Na is due to supernatant included above the cells, then the volume of this included supernatant:

$$\frac{7.5 \text{ Eq}}{3.5 \text{ Eq/l}} = 2.2 \mu\text{l}$$

Supernatant K concentration = 5 mM per liter  
= .005  $\mu$ Eq per  $\mu$ l

. . . Maximum possible amount of K in supernatant above the cells =  
(2.2) (.005)  $\mu$ Eq  
= .01  $\mu$ Eq

Since cell K = pellet K - trapped K

. . . cell K = 1.01 - .01  $\mu$ Eq  
= 1.00  $\pm$  0.02  $\mu$ Eq in cells in 250  $\mu$ l suspension  
. . . cell K = 4.00  $\pm$  0.08  $\mu$ Eq in cells/ml. suspension

### B. Total Na

Volume of bacterial suspension centrifuged in microfuge tube : 250  $\mu$ l

Cell pellet dispersed in 5 mls. distilled water

Na concentration : 167  $\pm$  4  $\mu$ Eq (mean of 4 samples)

K concentration : 149  $\pm$  1  $\mu$ Eq (mean of 4 samples)

$$(\text{Na}) / (\text{K}) = 1.12 \pm 0.3$$

$$\begin{aligned} \text{Amount Na in this volume} &= (1.8) (.256 \pm .008) (3.5) \mu\text{Eq} \\ &\text{(since Na concentration is } 3.5 \mu\text{Eq} / \mu\text{l)} \\ &= 1.64 \pm 0.05 \mu\text{Eq} \end{aligned}$$

$$\begin{aligned} \text{Cell Na} &= \text{Total Na} - \text{Trapped Na} \\ &= (4.48 \pm 0.12) - (1.64 \pm 0.05) \mu\text{Eq} \\ &= 2.84 \pm 0.13 \mu\text{Eq in cells in 1 ml. suspension} \end{aligned}$$

The amount of cell Cl is calculated in the same way and is  $4.44 \pm 0.09 \mu\text{Eq}$  in cells in 1 ml. suspension.

### Calculation of Cell Water

Cell water was calculated from the measured pellet volume according to the following formula :-

$$X = v (1 - V_s) (P_c) (1 - A/B)$$

X = cell water in mg.

v = measured volume of a given pellet. See "Measurements of cell mass, b) Volume".

P<sub>c</sub> = cell density.

The cell density is measured by preparing different mixtures of dimethylphthalate and octoil (Consolidated Vacuum Corporation, Rochester N. Y.). The specific gravity of the dimethylphthalate is 1.191 and that of the octoil 1.064. Both substances are miscible with each other and hence mixtures of known specific gravity can be prepared. The cell density equals the density of the mixture which permits the centrifugation through it of 50% of the cells.

At 37°C, rather more than half of the cells from logarithmic phase suspensions sedimented through pure dimethylphthalate. The average cell density was therefore slightly greater than 1.191. A value of 1.2 was therefore arbitrarily taken, since the error introduced by the uncertainty at the second decimal place is unlikely to affect the result by more than 5% of the total value.

$$\begin{aligned} \therefore \text{Total Na} &= (\text{Cell K}) ([\text{Na}] / [\text{K}]) \\ &= 4.48 \pm 0.12 \mu\text{Eq in pellet from 1 ml. suspension} \end{aligned}$$

### C. Total Cl

Same sample as for Total Na.

Amount of Cl in 3 ml. portion =  $0.827 \pm 0.007 \mu\text{Eq}$  (mean of 3 samples)

$$\therefore \text{Concentration} = 276 \pm 2 \mu\text{Eq}$$

K concentration after ashing =  $182 \pm 3 \mu\text{Eq}$

$$(\text{Cl}) / (\text{K}) = 1.52 \pm .02$$

$$\begin{aligned} \therefore \text{Total Cl} &= (\text{Cell K}) ([\text{Cl}] / [\text{K}]) \\ &= 6.08 \pm 0.08 \mu\text{Eq in pellet from 1 ml. suspension} \end{aligned}$$

### Calculation of Cell Na and Cl

It is first necessary to measure the amount of supernatant trapped within microfuge pellets, in order to allow for the relative contributions of supernatant and cells to total Na and Cl in these pellets.

The measurement of trapped supernatant in microfuge pellets has been described elsewhere (11). It was established that the volume of supernatant trapped within microfuge pellets after a centrifugation period of 5 minutes, was  $25.5 \pm 0.8\%$  (S. E.) of the total pellet volume obtained when the same suspensions were centrifuged in cytocrit tubes (mean of 45 determinations).

An example for the calculation of cell Na is given below.

Total Na =  $4.48 \pm 0.12 \mu\text{Eq}$  in pellet from 1 ml. suspension

Pellet volume =  $1.8 \text{ mm}^3 / \text{ml suspension}$

$$\therefore \text{Volume of trapped supernatant} = (1.8) (.256 \pm .008) \text{ mm}^3$$

### Specimen calculation

Pellet volume =  $1.8 \text{ mm}^3$  / ml suspension

Since  $32.1 \pm 0.5\%$  of the total pellet volume is occupied by supernatant,

the cell volume is  $1.22 \pm 0.02 \text{ mm}^3$

$$\begin{aligned}\text{Cell fresh weight} &= (1.22) (1.2) \text{ mg} \\ &= 1.46 \pm 0.024 \text{ mg}\end{aligned}$$

Weight of cell  $\text{H}_2\text{O}$  =  $(1.46 \pm 0.024) (.596 \pm 0.0014) \text{ mg}$ , since cell  $\text{H}_2\text{O}$  is 59.6% of cell fresh weight at this stage of growth (Table 2).

$$= 0.87 \pm 0.04 \text{ mg}$$

### Calculation of Ion Concentrations

#### Specimen calculations

$$\begin{aligned}\text{Cell K concentration} &= \frac{4.00 \pm 0.08}{0.87 \pm 0.04} \mu\text{Eq per mg cell H}_2\text{O} \\ &= 4.60 \pm 0.22 \text{ Moles per kg. cell water}\end{aligned}$$

$$\begin{aligned}\text{Cell Na concentration} &= \frac{2.84 \pm 0.13}{0.87 \pm 0.04} \mu\text{Eq per mg cell H}_2\text{O} \\ &= 3.26 \pm 0.2 \text{ Moles per kg. cell water}\end{aligned}$$

$$\begin{aligned}\text{Cell Cl concentration} &= \frac{4.42 \pm 0.09}{0.87 \pm 0.04} \mu\text{Eq per mg cell H}_2\text{O} \\ &= 5.08 \pm 0.26 \text{ Moles per kg. cell water}\end{aligned}$$

### Limits of Error in Calculating Cell Na and Cell Cl Concentrations

The error involved in the calculation of total Na and Cl is shown to be relatively small. A larger uncertainty is involved in the calculation of the trapped Na and Cl; the final mean cell Na has a Standard Deviation of 7% and mean cell Cl of 5% of the total amounts.

In the calculation of cell Na and Cl concentrations, the parameter of trapped volume is used twice, first for calculation of amount of cell ion and secondly for amount of cell water. Thus, any overestimation of the trapped space value would lead to a low estimate of cell Na and a low estimate of cell H<sub>2</sub>O, and hence a cell Na concentration closer than expected to the true value. The influence of trapped space on concentrations of Na and Cl is shown in Figure 1 for cells throughout the growth phase. In Figure 1 Na and Cl concentrations are calculated on the assumption that 20%, 30% or 40% of the total pellet volume is occupied by trapped supernatant. The 40% value is a clear overestimate since in the stationary phase, it leads to a calculated trapped Na larger than the total measured Na. The 20% and 30% estimates never differ by more than  $\pm 0.2$  Moles per kg cell water.

## R E S U L T S

### Dry Weight Content of the Bacteria

The dry weights contents of the bacteria at various stages of growth are described in Table 2. In cultures at an optical density of 0.2, the dry weight content was measured by both methods used (see Methods); these yield results of  $46.1 \pm 1.0\%$  (method 2) and  $49.8 \pm 0.3\%$  (method 1). The agreement between the two methods is probably within the limit of error of the methods used. In the midlogarithmic phase the dry weight content is at its lowest (40.4%) and increases at later stages of growth, reaching a value of  $45.4 \pm 1.2\%$  in the stationary phase. Christian and Waltho (1) found a dry weight of 50% for a related species at the same phase of growth.

The high dry weight contents of the bacteria can be accounted for by their high salt content.

## Growth of Culture of Halobacterium

The growth curves of the halophilic bacteria, typical of 17 cultures measured throughout the growth period, is shown in Figure 2. An initial inoculum of about  $10^7$  cells / ml. was used. Although the cultures were inoculated from mother cultures in the logarithmic phase of growth, there was an initial lag. The first measurements of optical density, protein content and volume of the cells were made 20-24 hours after inoculation. After this, growth proceeded logarithmically until 40 hours after the start of the culture. During this phase the generation time was 6.5 hours, as calculated from all the parameters measured. This appears to be the shortest generation time recorded for a Halobacterium: Larsen (7) has written: "The extreme halophiles multiply slowly even under the most favorable conditions designed. The shortest time obtained for the halobacteria is not much less than 7 hours."

A comparison was made of cell counts with a Petroff-Hausser bacteria counter and viable counts by dilution and plating on agar plates. It was concluded that in 60-hour cultures (stationary phase) at least 90% of the total cells yielded colonies.

The original pH of the medium was 7.0. For the first 28 hours of growth the pH fell, due presumably to the activity of the bacteria. The lowest value reached was pH 6.4. Thereafter, the pH rose to a final value of 7.5 which was reached at the end of the experiment.

The rates of oxygen consumption per unit of protein are shown in Figure 3. The oxygen consumption remained relatively constant throughout the logarithmic phase, despite changes in pH. When the growth rate decreased, the oxygen consumption fell to about a third of its initial rate.

## Ions in Growing Cultures

The total amount of ion in the cells in 1 milliliter of suspension are plotted in Figures 4a, 4b, 4c. From these figures can be calculated the net influx of ion during the growth phase. The net K influx is  $50 \times 10^{-12}$  Moles/cm<sup>2</sup> x sec. assuming a cell radius of  $0.5 \mu$ . This is 4-5 times higher than the net flux calculated from Schultz's results (2) for E. coli.

No net decrease in amount of cell K occurred in the stationary phase (Figure 4a). However, a net decrease in Na was observed at this time (Figure 4b).

Ion concentrations are shown in Figures 5a and 5b which are the results of 2 separate experiments performed in July 1968 and January 1969. An essential similarity of the 2 experiments is clear, although there are differences in timing. Except for the 20 and 24-hour points the K concentration stayed steady at  $3.7 \pm 0.2$  Moles per kg. cell water throughout the growth phase, even in the stationary phase at a time when the medium K concentration was under 1 mM/l.

The Na concentration is also seen to fall in the period from 20 to 40 hours of growth, and reaches minimum values of 1.2 Moles/kg. cell H<sub>2</sub>O in experiment 1 and 1.0 Moles in experiment 2. There is then a rapid increase to 3 M/kg. cell water (experiment 1) and 2.4 Moles (experiment 2), followed by a slow decline throughout the stationary phase to a final concentration of 0.5 M/kg. cell water, well below the medium Na concentration of 3.9 Molal.

The Cl concentrations follow the pattern of Na concentrations.

Figure 5 demonstrates that the sum of the Na + K concentrations is always greater than the Cl with an average anion deficiency of 2 Moles per kg. cell H<sub>2</sub>O. The PO<sub>4</sub> concentration was measured on 2 sets of samples in the logarithmic phase and was found to be  $125 \pm 25$  mM/kg. cell water. These results imply that excess cation charges must be balanced by organic anions.

Thus, ion concentrations in cultures of these halophilic bacteria are not characterised by any one set of values; even in cultures growing and respiring at steady rates there may be wide fluctuations in concentrations of Na and Cl. On the other hand, stationary phase cultures in which no net growth occurs are characterised by a K concentration nearly 5000 times higher than in the outside medium ( $K_{in}$  : 3.75 Moles/kg. cell water;  $K_{out}$  0.75 mM) and a Na concentration lower than in the outside medium. ( $Na_{in}$  0.50 Moles / kg. cell water; NaCl outside 3.9 Molal).

## DISCUSSION

The ion metabolism of Halobacterium sp. has been shown to bear some features of resemblance with that of other organisms so far studied. For example, Na, K and Cl are ions of major importance in the cell interior, as is the case in most other organisms. Furthermore, the cell concentrations of these ions differ from those in the medium, implying that metabolic, electric and other activities are involved in the regulation of the cell ion concentration. The distribution of these ions is summarized in Table 3. The nature - passive or active - of the ion fluxes will now be considered in the light of the potential difference which may exist across the cell membrane.

Should the P.D. be close to zero, the Cl ion would be distributed passively when the Cl chemical concentrations are equal on both sides of the membrane. K influx and Na efflux would have to be coupled to some metabolic reactions. Such a situation is found in many animal cells and in E. coli (1). It does not necessarily imply the existence of a Na-K exchange pump, nor do all the cell ions have to be in solution.

An alternative model assumes K to be passively distributed through the existence of a high potential (up to 200 mV, inside negative with respect to the

exterior). Such high P. D. have been found in *Neurospora* by Slayman (15). When such is the case, Na efflux must be active as before, but more energy is required to bring about the active efflux because the electrochemical potential gradient is higher than in the first model. Cl influx, in this second model, would be active. Active Na effluxes and Cl influxes have been detected in Nitella (16) and Chlorella (5), for example. This picture could apply to the metabolising Halobacterium cell.

If ion movements are indeed coupled to metabolic reactions which maintain gradients in ion concentrations, then the inhibition of metabolism should lead to the reduction and eventual abolishment of ion concentration gradients. This prediction has been generally fulfilled in most tissues and organisms studied up to now. Quite on the other hand, higher gradients of Na and K were observed in stationary state cells than in logarithmic phase cells even though the rate of respiration per unit of protein was lower. High gradients have been observed in experiments in which O<sub>2</sub> uptake was completely abolished (17). Thus it may be argued that, in Halobacterium, the mechanism of ion movements is not due to "active transport" reactions in the usual sense of the word.

There is another major difference between ion metabolism in Halobacterium sp. and that of most organisms so far studied. It appears to be the usual case that the cell ions, or the greater part of them, diffuse freely within the cell water (18). There are several types of evidence against this being the case in Halobacterium sp.

First, Table 3 shows that in growing cells large differences in total ionic content exist between the cell interior and the external medium. It follows that, if the cell ions were in solution, they would exert an osmotic pressure up to several hundred atmospheres during the logarithmic phase of growth. Thus a high hydrostatic pressure would be exerted against the cell envelope. For cells to tolerate so high a hydrostatic pressure, a rigid, mechanically-strong cell-wall would be needed. But, according to Larsen, who has summarized the evidence (7), the halophilic bacteria have no polysaccharide cell-wall.

It is generally agreed that the halobacteria are bounded by a lipoprotein cell envelope which fulfills the role both of cell-wall and cell-membrane. (7) This envelope differs from the cell-walls of most other bacteria in that it does not contain muramic acid, nor any other muco-peptide which might confer rigidity to the structure (19).

We have observed large changes in cell volume brought about by changes in medium pH (to be published). Since it is difficult to envisage a mechanically-strong cell wall which is also highly elastic, the results presented here make it likely that halophilic bacteria do not possess a rigid cell-wall and that there is little or no hydrostatic pressure across the envelope. In this case, a portion of the cell ions must have a reduced osmotic coefficient.

A second argument in favor of reduced ion activity coefficients comes from the high cell ion concentrations themselves. KCl solutions of 4-5 Molal are almost saturated; it is physically impossible to make an aqueous solution of 4-5 M KCl + 1 - 3 M NaCl. Yet these are the amounts of ion present in the bacteria. It can only follow therefore that the activity of a part of these ions is reduced.

The large amounts of cell K, together with the relative inertness of this ion in stationary phase cells and in the cold suggest that it is this cation, the activity of which is reduced.

Restricted K mobility is also suggested by the extreme permeability of the cell-membrane to moderately large molecules such as inulin and serum-albumin (11). It is hard to see how large amounts of K could be retained within so leaky a cell unless the K were specifically bound onto an intracellular molecule.

In conclusion, the Halobacterium cell has been found to consist of K-rich cytoplasm bounded by a permeable cell-membrane. The K is presumed to be retained within the cytoplasm by specific binding. Further evidence in favor of K-binding is presented later (17).

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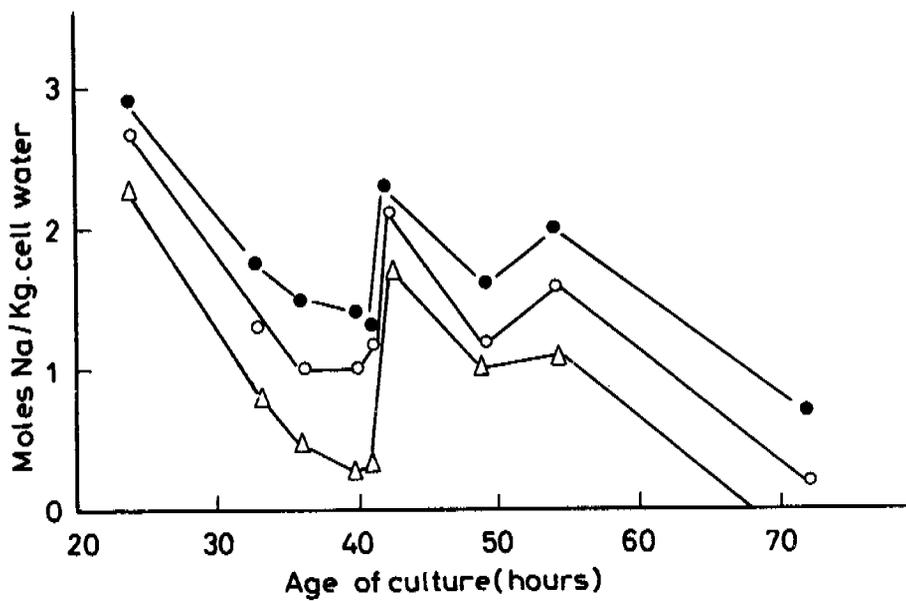
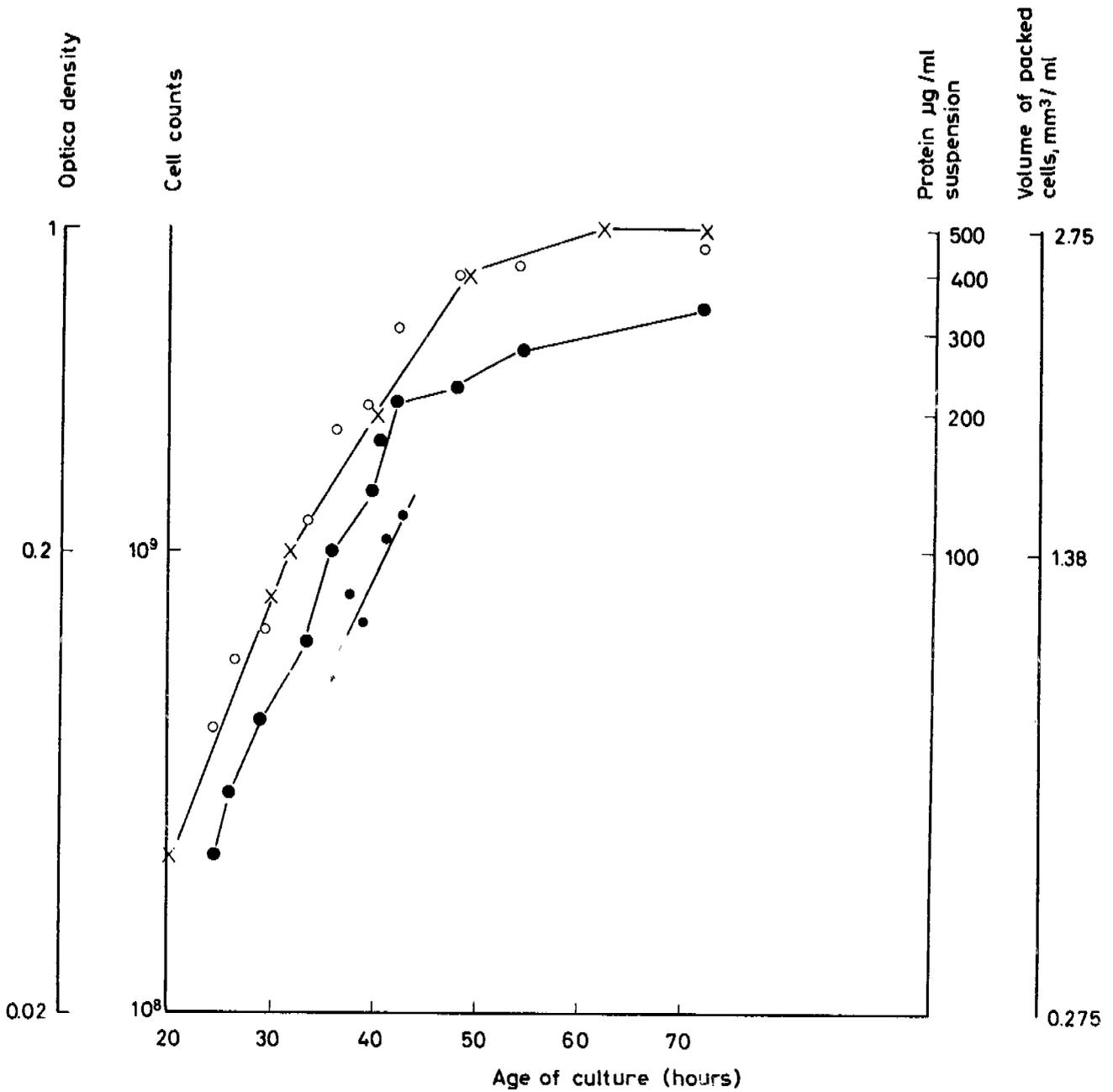


Figure 1.

Effect of value taken for calculation of trapped volume on Na concentration. ● : 20% of total volume assumed to be supernatant: ○ : 30%; △. 40%.



**Figure 2.**

Growth of Halobacterium sp. as measured by a) volume of cell material: x—x, b) total protein: o o. The line is drawn by joining the volume measurements. c) optical density: ● ● d) cell counts: • • . Initial pH of medium: 7.0. For composition of growth medium, see Table 1.

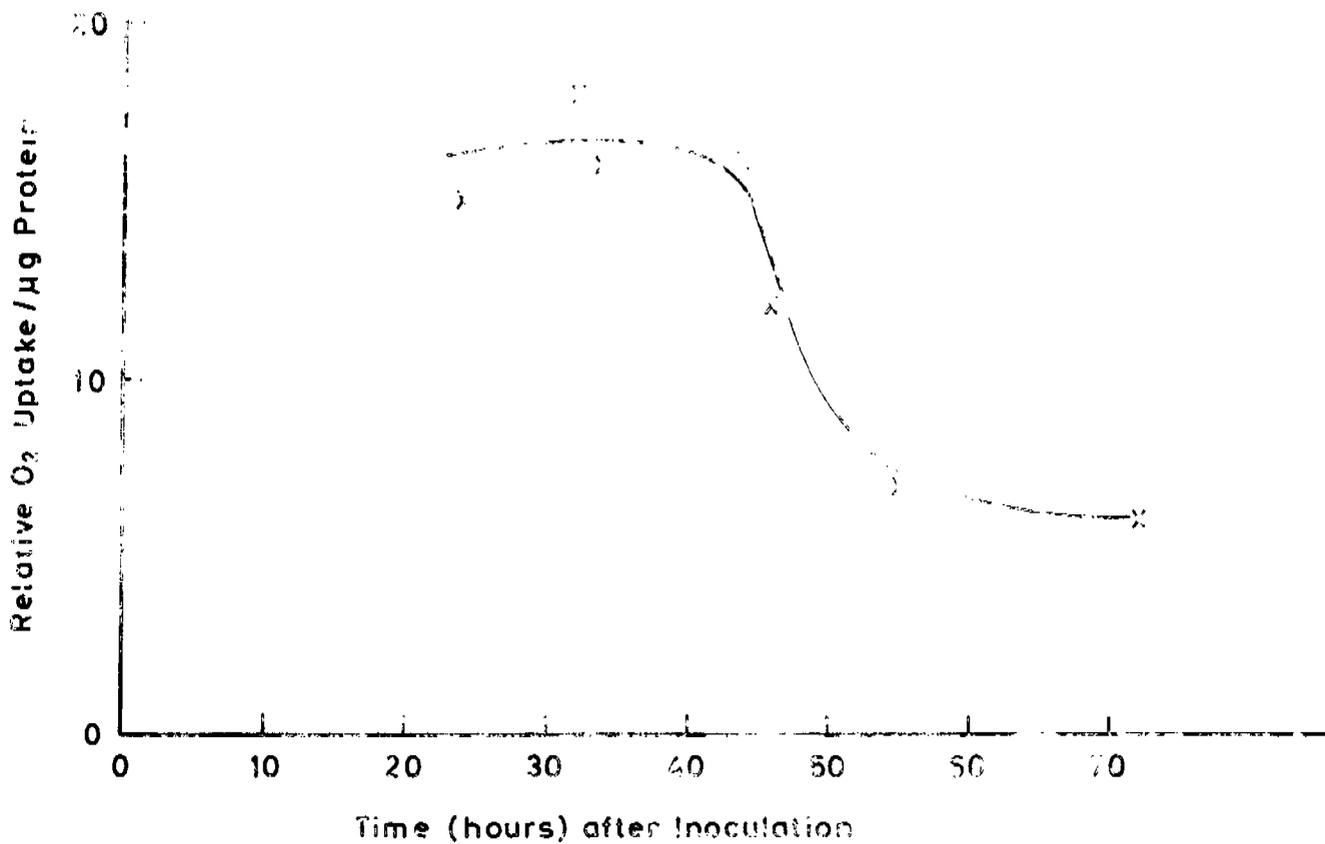


Figure 3.

O<sub>2</sub> consumption of Halobacterium sp. during period of growth.  
For conditions of culture, see legend to Table 1.

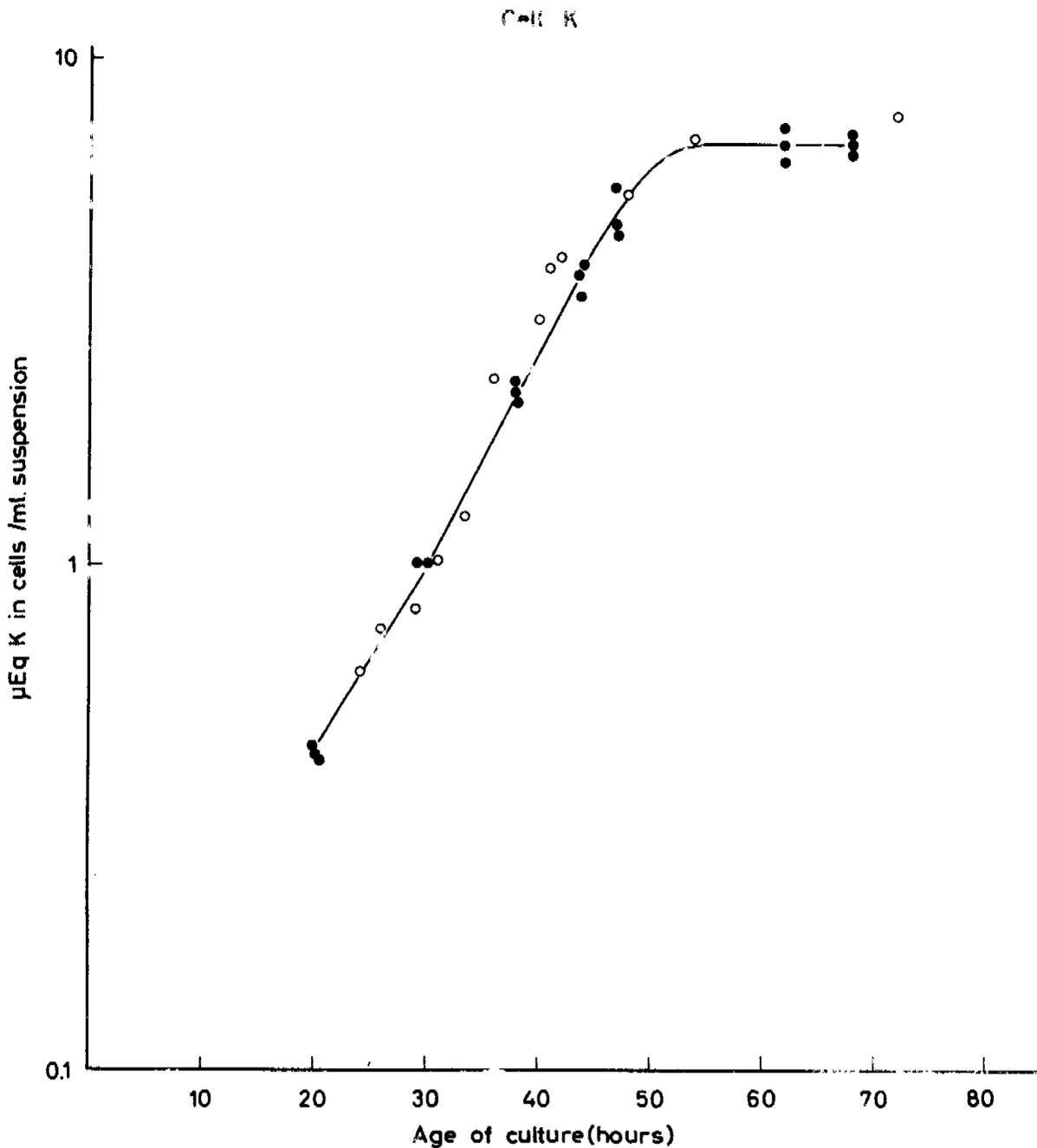


Figure 4.

Ion content of bacteria contained in 1 ml. suspension throughout period of growth. For conditions of culture, see legend to figure 2. a) K. b) Na. c) Cl. Open and closed circles refer to 2 separate experiments.

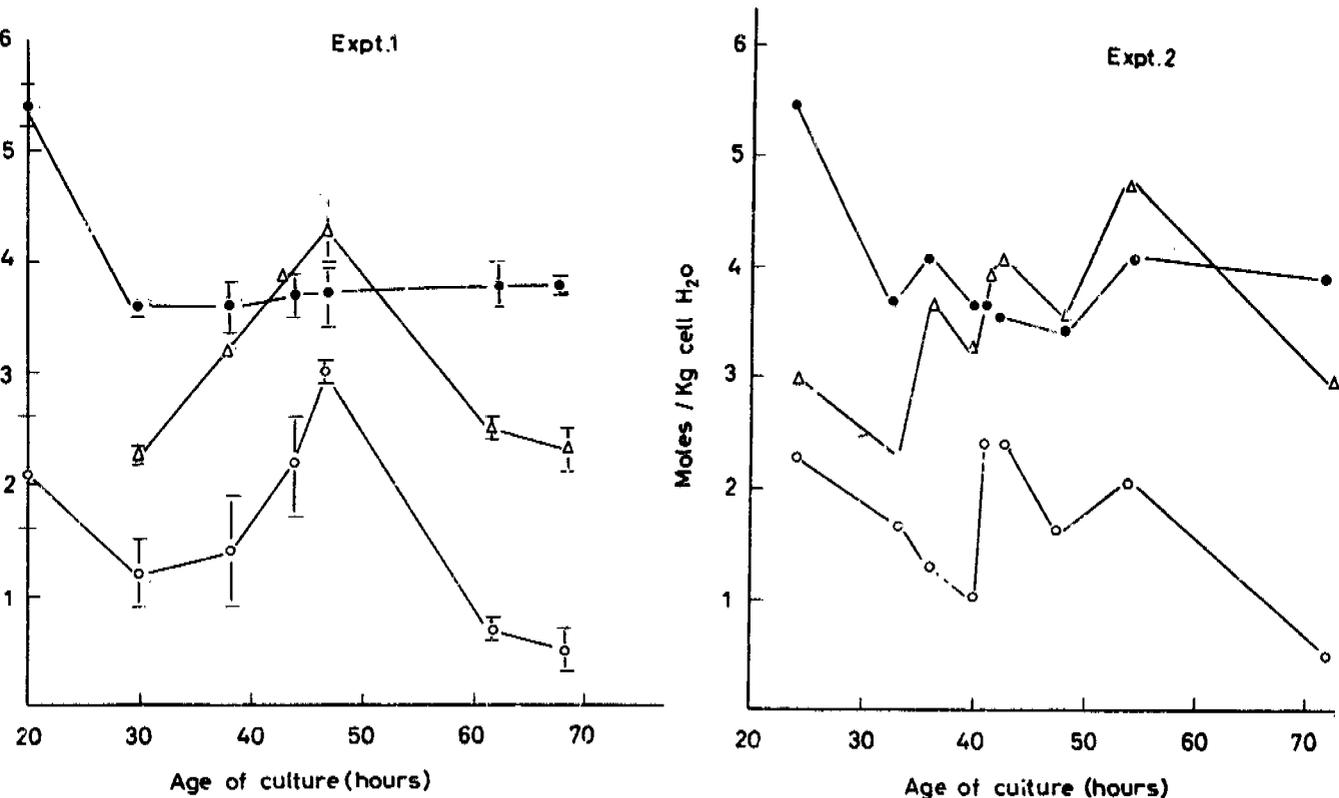


Figure 5.

Ion concentrations of Halobacterium sp. throughout growth phase. For conditions of culture, see legend to figure 1. Experiment A. Each point is the mean  $\pm$  Standard Deviation of 3 cultures measured at the same time. Experiment B. Each point represents a single culture selected randomly out of 11 cultures incubated together. ● : K, ○ : Na, Δ : Cl.

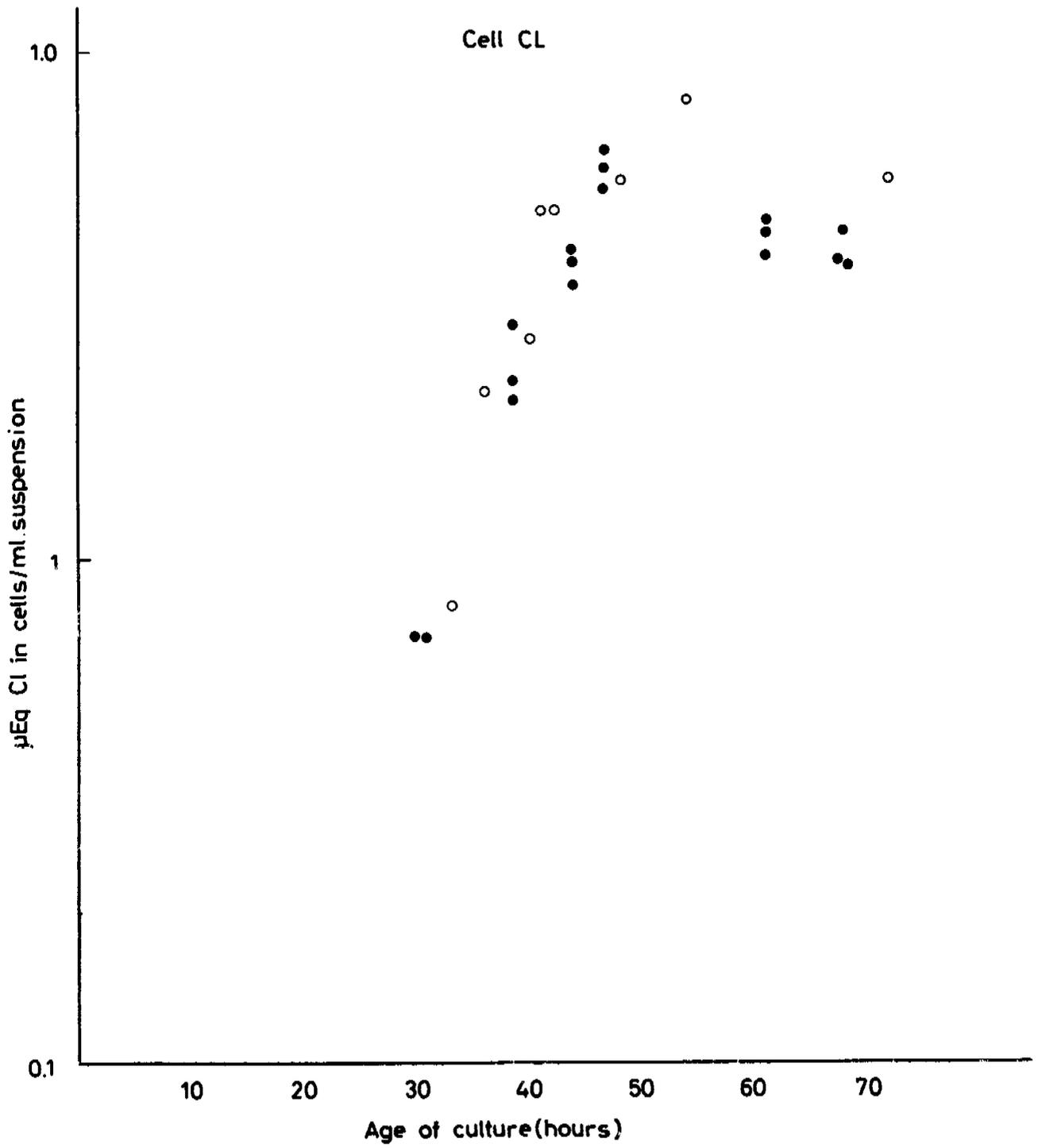


Table 1

Composition of growth medium

	<u>Millimolal</u>
Na <sup>+</sup>	3900
Mg <sup>++</sup>	150
Ca <sup>++</sup>	1.4
Mn <sup>++</sup>	$2.5 \cdot 10^{-4}$
K <sup>+</sup>	$7 \pm 0.5$
Cl <sup>-</sup>	3900
PO <sub>4</sub> <sup>----</sup>	1.25
SO <sub>4</sub> <sup>--</sup>	150
Yeast autolysate	10%
Initial pH : 7.0	

Table 2

Dry weight, as a proportion of total fresh weight  
of Halobacterium sp. at various stages of growth

Age of Culture		<u>% Dry Weight <math>\pm</math> S. E.</u>		<u>Number of Determinations</u>
<u>Hours</u>	<u>Method</u>	<u>Pellet</u>	<u>Cells</u>	
24	2	33.5 $\pm$ 0.3	46.1 $\pm$ 0.3	4
24	1	35.8 $\pm$ 1.0	49.8 $\pm$ 1.0	15
37	2	30.9 $\pm$ 0.14	40.4 $\pm$ 1.2	14
72	2	38.3 $\pm$ 0.8	45.4 $\pm$ 1.2	20

Table 3

Summary of biophysical parameters of Halobacterium sp.  
at different stages of growth

A. Ion concentrations, millimolal

K cell	3770 - 5500	3700 - 4000	3700 - 4000
medium	4 - 7	1 - 4	0.75
Na cell	1200 - 3000	1600 - 2100	500 - 700
medium	3900	3900	3900
Cl cell	2300 - 4200	3200 - 4100	2300 - 2900
medium	3900	3900	3900

B. Selectivity\*                      1000 - 3000                      2500 - 10,000                      33,000

C. Potential Difference (calculated)

K	-180	-200	-220
Na	(+31) - (+8)	(+23) - (+17)	(+54) - (44)
Cl	(-17.0) - (+2.5)	(-4.0) - (+2.0)	(-17.0) - (-7.0)

D. Osmotic Pressure

$\Delta c$	400 - 6100	00 - 2900	1100 - 0
$\Delta \pi$ , atmospheres (approximate)	-10 - 250	22 - 75	-27.5 - 0

$$* \text{ Selectivity} = \frac{(K_{in}) / (Na_{in})}{(K_{out}) / Na_{out}}$$

Effect of metabolism on ion concentrations  
of Halobacterium sp.

## INTRODUCTION

The classical experiments of Maizels in 1940 proved that ion fluxes were coupled to metabolic reactions in red blood cells. Since then, every kind of animal, plant or bacterial cell studied has demonstrated the important role of metabolism in the regulation of ion gradients across the cell membrane. It therefore seemed a logical starting place, in the investigation of ion metabolism in a Halobacterium species, to study with some thoroughness, the role of metabolism on the cell ions. Up to the present, this subject has been little investigated although measurements of ion concentrations in halophilic bacteria have been made on H. salinarium (1). The metabolism of the halobacteria is said to resemble that of other cell systems (2). The bacteria are highly aerobic (3). The metabolic pathways that have been investigated in detail, have shown that all enzymes so far isolated from halophiles have a requirement for 3-4 M KCl or NaCl (4, 5).

In the work to be described, the status of the  $K^+$ ,  $Na^+$  and  $Cl^-$  ions has been studied and contrasted in metabolising and in non-metabolising bacteria. Active protein synthesis and  $O_2$  uptake have been taken as essential criteria of metabolism; notice has also been taken of acidifying or alkalinising effects of the bacteria on the bathing medium.

## METHODS

Methods of culture of the Halobacterium sp. used have been described previously (6), together with methods of analysis and calculation.

## Metabolic experiments

Logarithmic phase cultures at an optical density of 0.20 were used. 70 mls. bacterial suspension were poured from the culture vessel into a jacketed, cylindrical vessel with an inner diameter of 8 cm. The vessel was covered with Parafilm. The depth of the suspension was 1.5 cm. It was rapidly stirred by means of a magnetic stirrer. A combined electrode (Radiometer GK264B), connected to a Radiometer Titrigraph TTTIC, dipped into the suspension and controlled the flow of  $N/5$  HCl into the vessel in response to alkalinization of the medium brought about by the bacteria. The response of the electrode used is not significantly affected by high salt concentrations below pH 9. The pH was carefully maintained at 7.0 in all experiments as the bacteria were found to be extremely sensitive to pH changes in the medium. The temperature of the suspension was maintained at  $37 \pm 0.2^{\circ}\text{C}$  by means of water pumped through the jacket of the vessel. A preliminary incubation period of 1 hour was allowed. This was followed by the experimental period which lasted from 1 to 5 hours.

## Incubation in argon

The experimental vessel was closed with a rubber stopper pierced with holes for the electrode and gas inlet and outlet.

The argon was passed through 2 water towers immersed in water at the same temperature as the cell suspension (either  $37^{\circ}\text{C}$  or  $0^{\circ}\text{C}$ ). The absence of oxygen from the experimental set-up was checked by means of an oxygen electrode (Yellow Springs Instrument Co. Inc) immersed in the cell suspension. In preliminary experiments a drop

of methylene blue was added to the suspension. It was entirely discolored 5-10 minutes after the argon was turned on. Ion measurements were not made on suspensions containing methylene blue.

### Resuspension experiments

The method of resuspension has been described previously (6). It took about 20 minutes for a button of cell material, obtained by centrifugation of a cell suspension, to be resuspended in fresh medium by the action of a rapidly-rotating magnetic bar. The first measurement was taken after the cells were completely resuspended and was designated as "time zero".

## R E S U L T S

### Control conditions

When cultures of Halobacterium at an optical density of 0.2 were incubated in the experimental vessel, there was an initial period of equilibration characterised by fast growth. This period lasted for one hour. During this time the parameters measured increased by 16% (mean of 10 cultures). In all of the figures given in this paper this initial period of equilibration is omitted.

After the period of equilibration, growth proceeded with a generation time of 5-7 hours (figure 6). This is the rate measured during the logarithmic phase (6). The changes in parameters measured - pellet volume, bacterial protein, K, Na and Cl during 3 hours of incubation in the experimental vessel - are shown in figure 6 .

Protein synthesis did not occur except under conditions as exactly described in the Methods; any interference with the aeration of the culture, as for instance, by having a larger volume of

suspension, or by changing the shape of the vessel, stopped both protein synthesis and K uptake. This supports the statement, made previously by others (3), that Halobacterium is an obligate aerobe.

#### Effect of cold

In 9 separate experiments bacterial cultures in the logarithmic phase were cooled to 0°C and maintained there for periods of up to 24 hours. They were vigorously aerated for the first 3 hours of the experiment, after which they were stored in a refrigerator overnight. The pH was maintained at 7.0. O<sub>2</sub> uptake was very low (not more than 3% of the rate at 37°C). It was interesting to observe that when the temperature was dropped from 37°C to 0°C, the bacteria ceased to alkalinise the medium and started to acidify it. This acidification lasted for the duration of the experiment, but at greatly reduced rate after the first hour. There were no increases in protein or K during the experimental period (the amounts after 24 hours were 97% and 104% for protein and K respectively, of the initial values). On the other hand protein synthesis and alkalination of the medium were resumed immediately on returning the temperature to 37°C, even after 24 hours at 0°C. Bacterial protein, after 1 hr at 37°C following 24 hours at 0°C, increased by 10% of the original amount.

It should be remembered that the bacteria and their medium are far from their freezing point. The freezing point for 3.5 Molar NaCl is -20°C.

The effects on ions are summarised in figure 7. The cell potassium concentration fell by 10.5% after 3 hours in the cold

(initial:  $4.8 \pm 0.2$  Moles/kg. cell water; after 3 hours at  $0^{\circ}\text{C}$ :  $4.3 \pm 0.2$  Moles/kg. cell water). The final 24-hour value of  $4.45 \pm 0.2$  Moles/kg. cell water was not, however, significantly different from the initial concentration. It is concluded that exposure to cold does not reduce cell potassium concentration.

Large effects of cold were found on cell Na and Cl. Initial concentrations were:  $2.8 \pm 0.2$  Moles Na and 5.5 Moles Cl per kg. cell water. During the first hour a slight increase to  $3.2 \pm 0.1$  Moles Na/kg. cell water was measured. This increase took place suddenly 20-40 minutes after the exposure to cold. As time went on, cell Na was lost; 3 hours after the exposure to  $0^{\circ}\text{C}$  began the cell Na concentration reached  $1.5 \pm 0.03$  Moles Na per kg. cell water. The 24-hour value of  $1.25 \pm 0.15$  M. Na per kg. cell water is even lower. The loss in cell Na was accompanied by a parallel loss in cell Cl; thus the total losses were  $1.5 \pm 0.23$  Moles Na and  $1.75 \pm 0.23$  Moles Cl which were lost simultaneously.

It is concluded that cooled cells are characterised by K and Na gradients across the cell membrane. The K gradient is 1:890 ( $K_{\text{out}}: 5\text{mM}/1_i K_{\text{in}}: 4.45$  Moles/kg. cell water) and the Na gradient 3.2:1 ( $\text{Na}_{\text{out}}: 3.9$  Molal;  $\text{Na}_{\text{in}}: 1.25$  Moles/kg. cell water).

In a second series of experiments 5 bacterial cultures were vigorously stirred at  $2^{\circ}\text{C}$  for 24 hours. Results in Table 4 show that no protein synthesis occurred during this time, as in the experiments described in Figure 7; there were, however, significant increases in the amount of cell K and in cell volume. ( $144 \pm 2\%$  and  $114 \pm 1\%$  of the original values, respectively).

Thus the cell K concentration actually increased during this time.

The pH was not controlled during this latter series of experiments. As comparison, are given the results for cultures aerated only during the initial 3-hourly period. These cultures showed no increase in amount of cell K. The results demonstrate that aeration is essential for K uptake, just as higher temperatures are essential for protein synthesis.

### Incubation in argon

In cultures treated with argon there was no protein synthesis. In fact, after the first 3 hours of treatment, the cell protein was  $97 \pm 1\%$  of its initial value. Thereafter, a slow decrease was found, likely due to lysis, so that after 5 hours in argon, cell protein was  $89 \pm 1\%$  of the original amount.

Incubation in argon caused a loss in cell K (figure 7). The lines in figure 2 are fitted by the least-squares method. The losses in K occurred in a linear manner both at  $37^{\circ}\text{C}$  and at  $0^{\circ}\text{C}$ .

The curves representing loss of K do not fit the usual kinetics of one-compartment systems and are evidence against the cell K being present in solution within the cell. This will be discussed at greater length later.

The fall in concentration of cell K at  $37^{\circ}\text{C}$  in argon is shown in figure 8. The line through the K points is fitted by the least squares method. The other 2 curves are drawn by joining the points. The Na concentration did not change significantly during the period of measurement. The Cl concentration, however, did fall

slightly. Thus the loss of  $2.0 \pm 0.3$  Moles K per kg. cell water which occurred during the first 4 hours of the experiments was accompanied by a loss of  $0.8 \pm 0.2$  Moles Cl per kg. cell water. The ratios of K:Na:Cl (see Table 6) were <sup>not</sup> the same after 4 hours incubation in argon as at the beginning. Cultures incubated in argon were observed to bring about alkalisation of the medium. It is therefore possible that a portion of the K was lost together with  $\text{OH}^-$  or was exchanged for  $\text{H}^+$ .

When cultures were incubated at  $0^\circ\text{C}$  in argon the concentrations of Na and Cl fell (figure 9). The rates of loss of Na and Cl were faster and the final values lower in argon than in air (cf. figures 7 and 9).

The ion gradients in cells after 5 hours at  $0^\circ\text{C}$  in argon are K:- 1:660 ( $\text{K}_{\text{out}}$ : 5mm,  $\text{K}_{\text{in}}$  3.3 Moles per kg. cell water) Na:- 10:1 and Cl:- 1.65:1 ( $\text{NaCl}_{\text{out}}$ : 3.9 Molal;  $\text{Na}_{\text{in}}$ : 0.4 Moles and  $\text{Cl}_{\text{in}}$ : 3.4 Moles per kg. cell water).

#### Deprivation of substrate

On resuspending bacteria from cultures at an optical density of 0.25 in saline solution in absence of any source of carbon or nitrogen, Halobacterium sp. cultures were found to lose potassium rather slowly. The composition of the saline solution was 3.9 Molal NaCl, 5mM KCl; 1.4mM  $\text{CaCl}_2$ ,  $2.5 \cdot 10^{-4}$  mM  $\text{MnCl}_2$ . At the end of 24 hours incubation in saline at  $37^\circ\text{C}$ , during which time the cells were continuously aerated and the pH maintained at 7.0, the cell ion concentrations were:- K:  $3.0 \pm 0.05$  Moles/kg. cell  $\text{H}_2\text{O}$ ; Na:-  $1.14 \pm 0.1$  Moles/kg. cell  $\text{H}_2\text{O}$ ; Cl:-  $2.48 \pm 0.06$  Moles/kg. cell

H<sub>2</sub>O. These are the mean of 5 experiments for K and Na, and 3 for Cl. That the low concentration of K is due to loss in amount of cell K, rather than cell swelling, is shown by the low K:protein ratio (see Table 5).

The ion concentrations of bacteria from cultures at an optical density of 0.06, after 24 hours incubation in saline, were:-  
K :  $3.85 \pm 0.05$  M.; Na :  $1.3 \pm 0.2$  per kg. cell water (means of 2 experiments).

Cells suspended in saline continued to absorb O<sub>2</sub> and to alkalise their medium for the first 2 hours after their resuspension in saline. Protein synthesis stopped at about this time. Afterwards, neither was O<sub>2</sub> uptake detected with the O<sub>2</sub> electrode nor did any change in pH of the medium take place. These were taken as signs of absence of any metabolic activity.

#### Resuspension of starved cells in complete medium.

The complete medium was similar in composition to the saline solution except for the addition of 10% yeast autolysate.

The bacteria initially responded to resuspension in complete medium by vigorous acidification of the medium. At some stage in the course of the ensuing 4 hours - generally associated with the time at which protein synthesis started - the bacteria started to make the medium alkaline. The pH was maintained at 7.0 by addition of small quantities of acid or base, as required.

Bacteria resuspended in complete medium absorbed oxygen throughout the experimental period at a rate comparable to that of bacteria in the logarithmic phase.

The courses of protein synthesis and volume increase in bacteria with a cell K concentration of 3.0 Moles per kg. cell water are shown in figure 11, curves A and C. There was no increase at all in cell protein for the first hour and only a slight one for the next 2 hours. For the final hour of the experiment, however, the increase in cell protein was such that, if continued, there would be a doubling of amount in about  $3\frac{1}{2}$  hours. This rate of increase would probably not be maintained for long.

The early lag phase is marked by a slight degree of cell swelling (figure 11, curve C). Curve B shows for comparison the rate of protein increase in bacteria with an initial K concentration of 3.85 Moles per kg. cell water; the average generation time over the 3-hour period is  $5.5 \pm 0.2$  hours. There was no initial lag.

Resuspension in complete medium led to a rapid uptake in amount of K, no matter what the initial cell K concentration (see figure 12). Increases in cell K concentration are shown in figures 8A and 8B. The final K concentrations were  $4.9 \pm 0.1$  Moles and 5.0 Moles per kg. cell water (low and high initial K, respectively).

Uptake of cell Na and Cl in bacteria with low initial cell ion concentrations are shown in figure 12. There was no significant Na uptake until the final hour of the experiment. At this time the Na concentration increased from 1.5 Moles to 2.4 Moles per kg. cell water (figure 8A). It was in the final hour of the experiment that protein synthesis was accelerated (figure 11 curve A).

It should be noticed that the highest K and Na concentrations measured in these resuspension experiments (for example, those shown in figure 13.), are close to those measured at the onset of the logarithmic phase of growth (6).

A second point of interest is that a fast rate of protein synthesis is associated with a high cell K concentration; in the cells with low initial K concentration, the rapid phase of protein synthesis started when the bacteria had a K concentration of  $4.45 \pm 0.1$  Moles per kg. cell water, whereas in the cells with high initial K concentration protein synthesis started at once (initial K concentration 3.85 Moles/kg. cell water). These observations agree with those of a number of workers who have found that enzymes isolated from halophilic bacteria require a high K concentration in order to be active (5).

#### Effects of metabolic inhibitors.

In contrast to the clear effects obtained by major changes in the environment (e.g. temperature,  $O_2$ , substrate), effects on ions due to metabolic inhibitors have been variable.

NaCN was tested in 14 experiments. This inhibitor arrested  $O_2$  uptake: 50% inhibition was obtained with 1mM/l. NaCN (figure 14). No protein synthesis was observed in cells treated with NaCN at 1mM or above.

Cell K fell steadily at about the same rate as in argon (see Table 7). No effect on cell Na was obtained. A marked, but variable effect was observed on cell Cl, the amount of which fell by

11  $\pm$  2% per hour. The overall effect was to increase the K:Cl<sub>K</sub> ratio (see Table 6).

No effects of ouabain have been detected in this system. Ouabain was tested at concentrations from 1 to 5 mm.

### DISCUSSION

In the cells and tissues of which the ion metabolism has been studied, ions have been found to cross the cell membrane in one of 2 ways. Either they follow the electrochemical potential gradient by passive means, or else their movements are coupled to chemical reactions. When the sources of chemical energy are removed, then passive flows are the only ones to occur and the ion concentrations inside the cell ultimately approach those in the medium outside.

We have taken as criteria for metabolism:- a) the occurrence of growth, as measured by protein synthesis, b) O<sub>2</sub> uptake, c) acidification or alkalisation of the medium as a result of bacterial activity. When all 3 criteria are negative, we say that Halobacterium cells are not metabolising.

Cells in which the absence of metabolism has been thus defined have been found in only 1 case, namely in cells suspended in saline. Such bacteria neither caused changes in pH of the medium nor respired nor synthesized protein, and according to the usual criteria any active transport processes should have ceased, so that after a lapse of time there should have been equilibration of the ion concentrations across the cell membrane. However, as we have seen, the ion concentrations of bacteria incubated for 24 hours in saline

were:-  $K_{in}$  3.0 Moles,  $Na_{in}$ : 1.14 Moles, Cl:2.48 Moles per kg. cell water ( $K_{out}$ : 5mM,  $NaCl_{out}$ : 3.9 Molal).

Protein synthesis was completely arrested in 2 other cases, namely in cells incubated at 0°C or in argon (see Table 4 and page 5 ). Oxygen uptake could not occur in bacteria incubated in argon and was much reduced in the cooled bacteria (page 76 ). It follows that the rate of metabolism must have been much reduced, if not entirely abolished under these conditions. Yet here again, equalisation of ion concentrations did not take place: the K concentration remained at nearly 1000 times its outside value and the cell Na concentration was always lower than that of the medium (e.g. 1.2 Moles/kg. cell water in cells at 0°C in air; 2.2 Moles/kg. cell water in cells in argon at 37°C as compared with 3.9 Molal NaCl in the medium). It has been shown previously (6) that in bacteria in the stationary state there exist the same high gradients of Na and K. In contrast, stationary state bacteria of *E. coli* are characterised by cell K and Na concentrations approximating to those in the outside medium (7).

Thus, in non-metabolising Halobacterium equalisation of the cell ion concentrations with those of the medium did not occur, and the cell ion concentrations must have been maintained at values widely apart from those in the medium by forces other than those due to active transport, as the expression is usually understood in the cells studied up to now.

Are ion concentration gradients in Halobacterium affected in any way by metabolic processes? It has been shown in this paper

that in cells metabolising according to the 3 criteria previously mentioned the highest cell ion concentrations were:- K:5.0 Moles, Na:2.45 Moles, Cl:5.15 Moles per kg. cell water (Figure 13). Thus the concentrations of all 3 major ions are high. It is concluded that metabolism plays a part in the regulation of ion concentrations, but does so by means other than those used in mesophilic tissues.

We are faced with several apparently unrelated problems, such as reactions controlling net uptake of K and its retention within the cell, and secondly, the reactions controlling the net fall of NaCl and the maintenance of low Na concentrations.

Let us first consider K uptake. The data have demonstrated that K uptake occurred in bacteria incubated with an organic substrate and oxygen (figure 6). Conversely, K was lost in bacteria incubated in saline solution or with argon (page 59 & figure 8). K influx was not inevitably accompanied by protein synthesis (Table 1; also compare protein synthesis and K uptake in figures 12 and 11, curve A). Thus, the chief conditions for K uptake in Halobacterium in 3.9 Molal NaCl are oxygen and an organic substrate. These conditions demonstrate that K uptake is an active process, requiring energy.

We have already seen that metabolic energy is not required to maintain a moderately high K concentration gradient, even though these bacteria are highly permeable (9). The lack of a requirement for metabolic energy to maintain a moderately high K concentration gradient is an argument in favor of the binding of K. Further evidence favoring this is given by the linear nature of the disappear-

ance of K from cells incubated in argon (figure 8); if the K were in solution within these highly permeable bacteria, the loss of K should have been logarithmic. These 2 lines of evidence, together with the arguments presented earlier for ion-binding (6), suggest that the cell K is bound to an indiffusible compound.

The determination of the K-complex requires analytical research. Information as to its nature may, however, be obtained by considering the electroneutrality of the bacteria. Analyses have invariably shown some anion deficiency of around 2 Moles per kg. cell water (Table 6). The anion deficiency must be made up by organic anions within the cell interior. If it is accepted that the cell Na and Cl are in solution, it is clear that these ions balance each other electrostatically. However, more Cl is present than is required to balance the Na and it is convenient to assume that the excess Cl is used to balance a portion of the cell K. This latter portion of the K is referred to as  $K_{Cl}$  in Table 6 and is the difference between the Cl and Na concentrations. In Table 6, cultures are grouped according to their metabolic activity, in group C (bacteria with little or no metabolic activity)  $K_{Cl}$  is inversely proportional to the cell Na concentration from 0.4 to 4 Moles per kg. cell water.

A number of organic compounds have recently been shown to form specific complexes with different alkali-ions (8). Examples of this type of compound are given by valinomycin and related neutral antibiotics. These complexes take on the charge of the complexed ion and are electrically neutralised by an external anion. This

type of compound would seem to fit the description of our  $K_{Cl}$  compound, which would thus act as a source of positive fixed charges within the cell. A second group of alkali-ion complexes form electrically-neutral ion-pairs with an alkali ion, after deprotonation. It may be that that part of the K not balanced by Cl within the halophilic bacteria, is complexed within a compound of this type. It is not suggested that the bacteria are loaded with compounds of the antibiotic type, but rather that radicles from the bacterial proteins form "cages" (within which the cell K is complexed) of the sort made familiar to us from work on valinomycin.

It is possible to explain the behavior of the cell Na and Cl by reference to the Gibbs-Donnan equilibrium, provided that one postulates the existence of positive fixed charges within the cell. We have already indicated that a portion of the cell K may indeed provide a source of positive fixed charges and have designated this portion of the K as  $K_{Cl}$ . In figure 15 are plotted concentration of positive fixed charges against cell Na concentration, calculated by means of the Donnan equation:

$$(m_+^o)^2 = \left[ \frac{\gamma_{\pm}^{in}}{\gamma_{\pm}^{out}} \right]^2 m_+^{in} (m_+^{in} - Z_{mp})$$

$$(m_-^o)^2 = \left[ \frac{\gamma_{\pm}^{in}}{\gamma_{\pm}^{out}} \right]^2 m_-^{in} (m_-^{in} - Z_{mp})$$

$m_+^{in}, m_+^o$  = Molality of cation inside and outside the cell, respectively.

$m_-^{in}, m_-^o$  = Molality of anion inside and outside the cell, respectively.

$\gamma_{\pm}$  = Mean ion activity coefficient.

Z = Valency of fixed polyelectrolyte.

mp = Molality of fixed polyelectrolyte.

B is the ratio of mean ion activity coefficients inside and outside the cell. In ideal solutions  $B = 1$ , and there is an ideal Gibbs-Donnan equilibrium. Figure 15 (points 1-6) indicates the concentrations of  $K_{Cl}$  and Na found in growing cells; under these conditions B is slightly above unity. Should B stay at this value, it would require an impossibly high fixed-charge concentration to account for the low Na concentrations found in cells with a low rate of metabolism. If, on the other hand, there were to be an increase in the ratio of the mean activity coefficients, i.e. B., there could be a fall in Na concentration without any increase in the concentration of the fixed charge. A change in activity coefficient could be brought about by local differences in the density of the electric fields, following some conformational changes in the proteins to which the positive fixed charges are bound.

The hypothesis is strongly supported by the results on ion concentrations in non-metabolising cells. Table 6, groups B and C, lists all the ion concentrations found by us <sup>in</sup> Halobacterium sp. after the treatments described in this paper. Stationary-state cells are included from the preceding paper (6). Ion concentrations

from cultures equilibrated at different pH values are also included (10). The figures of Table 6, group C are plotted in Figure 15 and demonstrate the inverse relation between  $K_{Cl}$  and Na concentrations over a tenfold range of Na concentration: the relationship, however, is not linear but involves a fall in B with increasing Na concentration.

In figure 16. are plotted total Cl concentration against Na concentration. The solid lines represent values calculated for different values of B. experimental results are also shown. The figure demonstrates that the observed equimolecular losses of Na and Cl are in agreement with the hypothesis that changes in concentration of Na and Cl are accompanied by changes in value of B. It should be noticed that the experimental points fall at the same value for B in figures 15 and 16. Such agreement further supports the hypothesis that ion concentrations in Halobacterium sp. are explicable in terms of Gibbs-Donnan equilibria modified by changes in the activity coefficients of the measured ions. It is worth noticing that this hypothesis, depending as it does on the postulation of fixed positive charges, favors the specific binding of K as compared with Na. Such an equilibrium system is capable of maintaining the cell ion concentrations at their given values even in the absence of an impermeable membrane. It has, indeed, been found that Halobacterium sp. has a highly permeable membrane (9).

The Halobacterium sp. cell is apparently a system in which ions are controlled by means of changes taking place within the cell

interior. This system requires energy firstly for uptake of K and its presumed binding with organic radicles to form a positively-charged complex and secondly for the maintenance of the cell proteins in a conformation suitable for metabolism.

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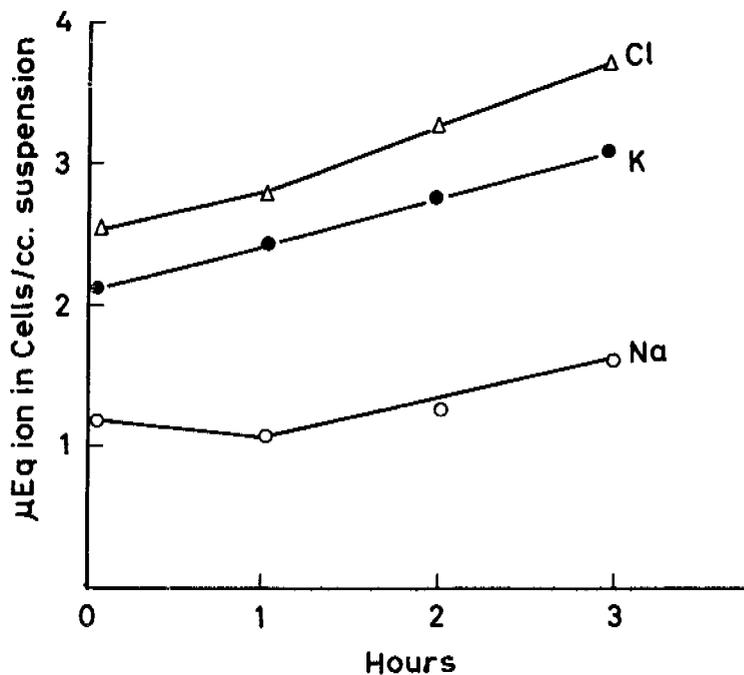
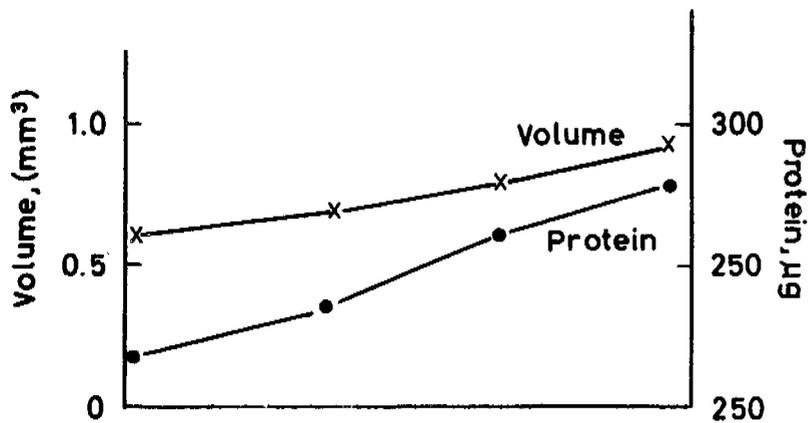


Figure 6

Growth of Halobacterium sp. cultures with C and N substrates at 37°C with vigorous aeration. Optical density: 0.2 Cell ion concentrations:  $4.75 \pm 0.05K$ ,  $2.34 \pm 0.2 Na$ ,  $5.0 Cl$ , all in Moles per kg. cell water. Medium NaCl concentration: 3.9 Molal. pH of incubation medium maintained at 7.0. For composition of medium see ref. 6.

● : K,    ○ : Na,    Δ : Cl.

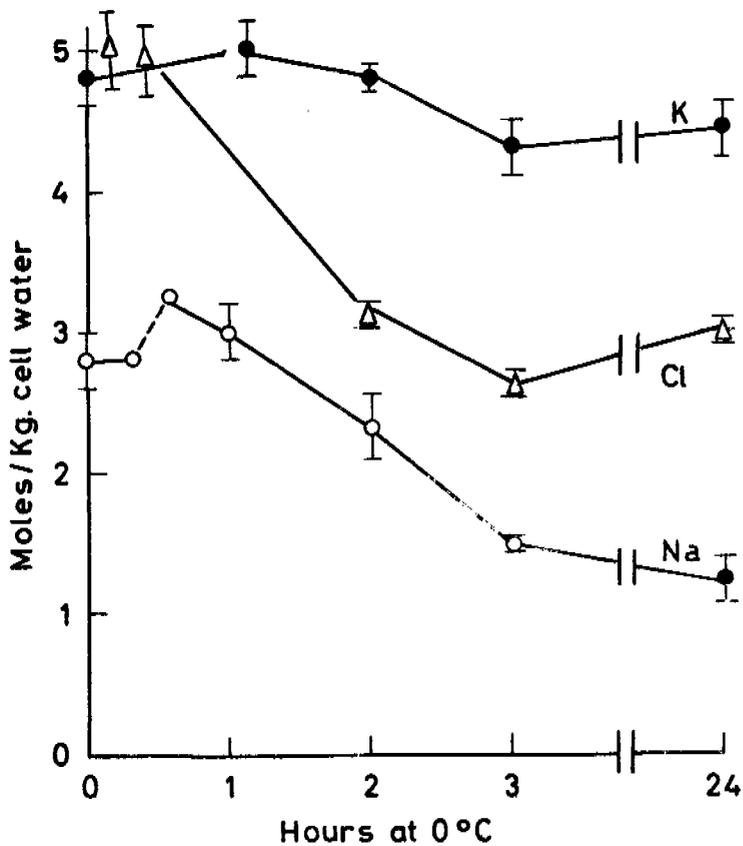


Figure 7.

Effect of incubation at 0°C on cell ion concentrations of Halobacterium sp. The cultures were aerated for the first 3 hours. For composition of medium see ref. 6. pH of incubation medium maintained at 7.0. Mean of 7 experiments.

● K, ○ Na, △ Cl.

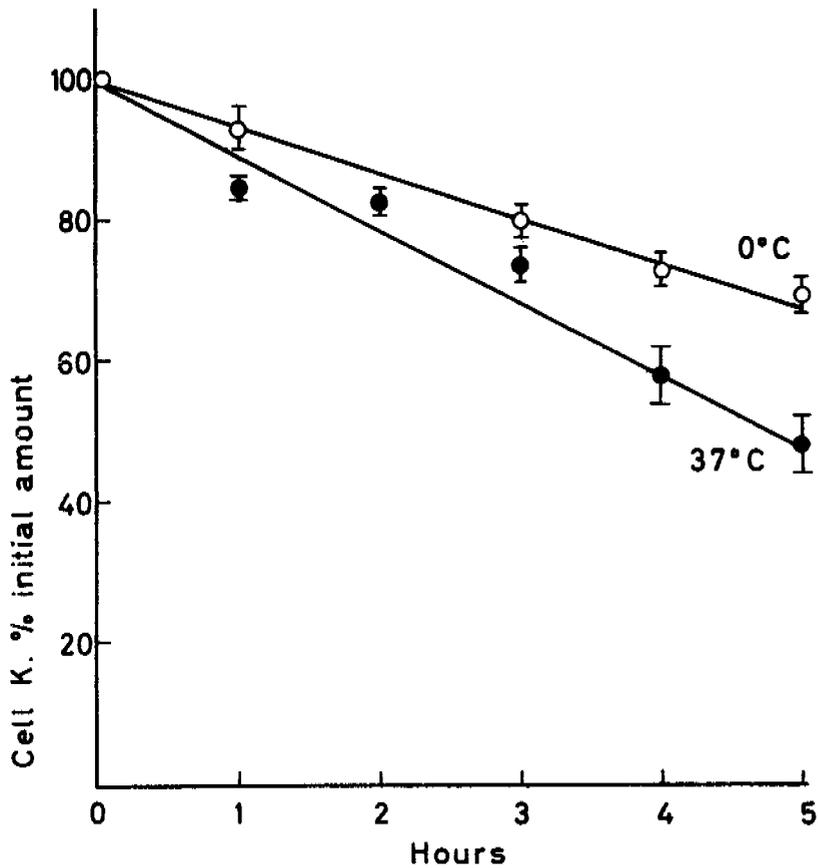


Figure 8.

Effect of incubation under anaerobic conditions on amount of cell K of Halobacterium sp. Anaerobic conditions obtained by passing a stream of argon through the cultures. For composition of medium see ref. 6. pH of incubation medium maintained at 7.0. Mean of 5 experiments.

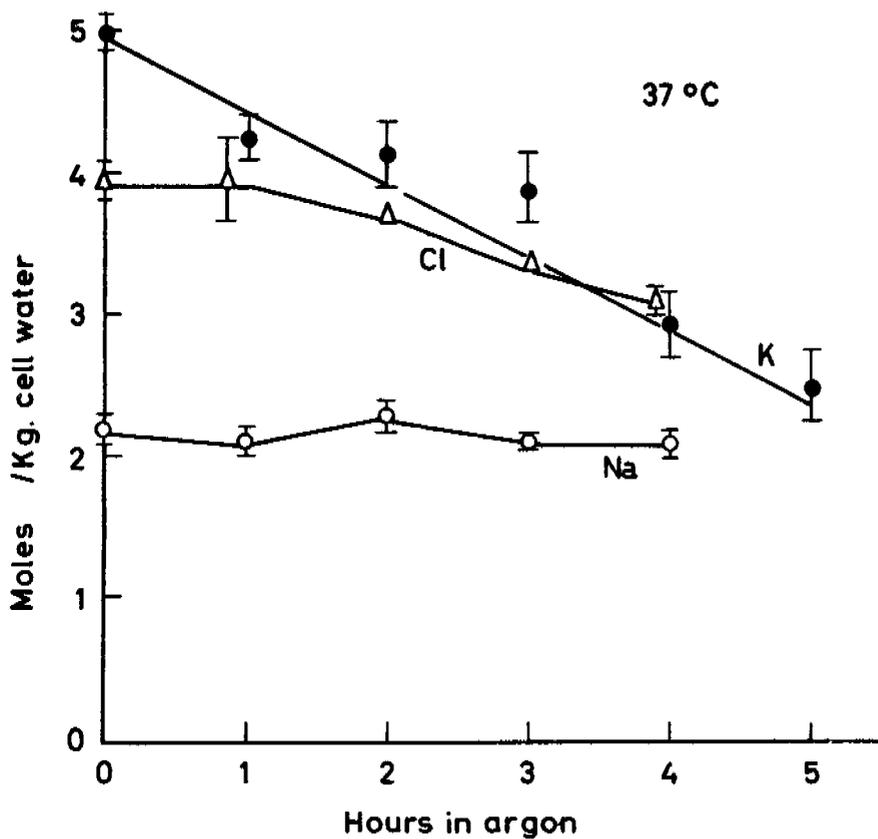


Figure 9

Effect of incubation under anaerobic conditions on cell ion concentrations of Halobacterium sp. Temperature of incubation: 37°C. For other conditions see legend to Figure 3.

● : K,    ○ : Na,    △ : Cl.

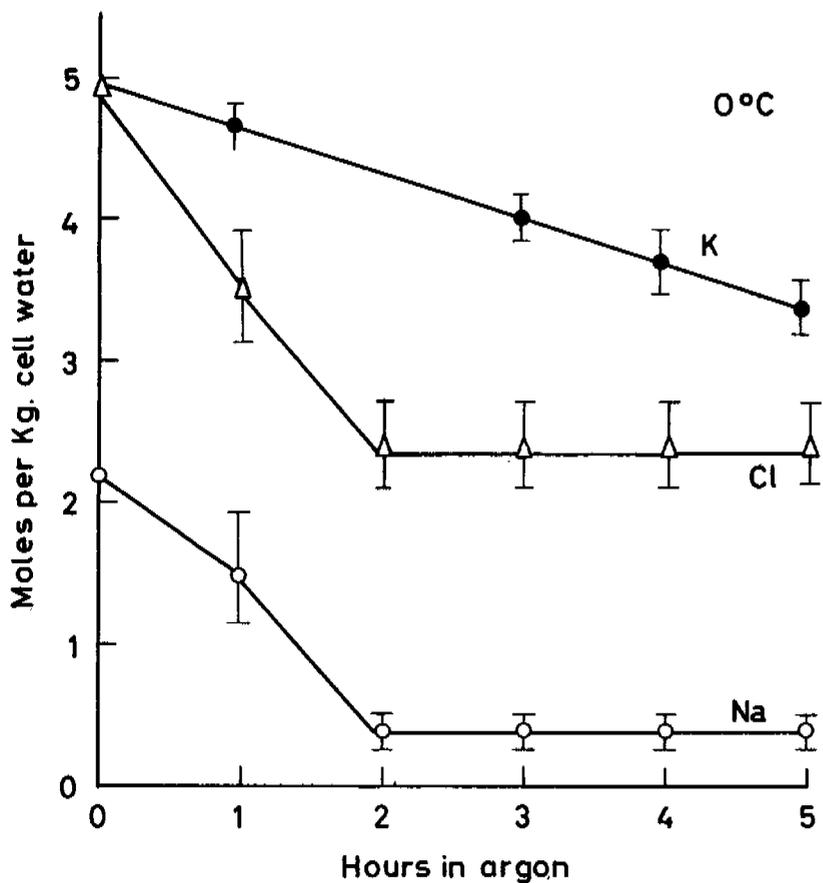


Figure 10

Effect of incubation under anaerobic conditions on cell ions of Halobacterium sp. Temperature of incubation: 0°C. For other conditions, see legend to figure 3.

● : K, ○ : Na, △ Cl.

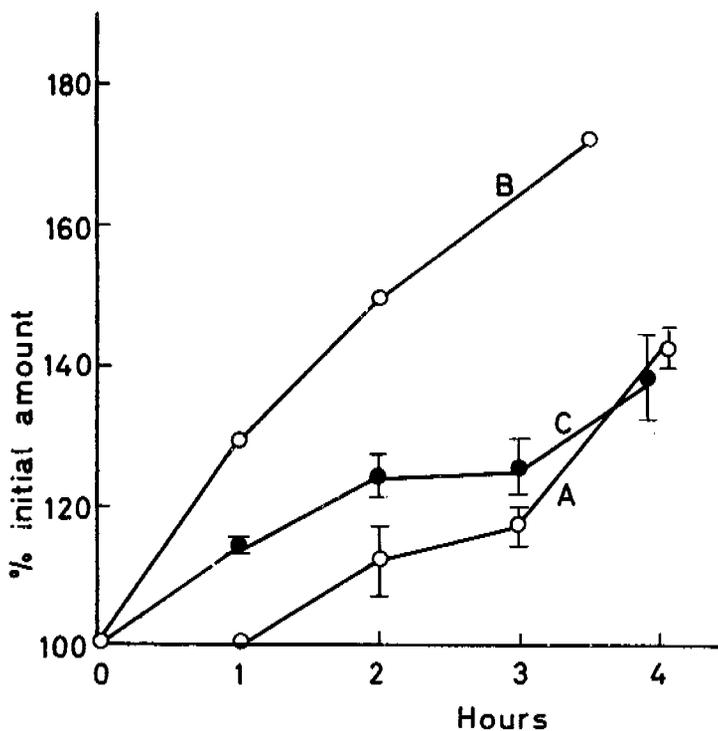


Figure 11

Increases in volume and protein in Halobacterium sp. cultures resuspended in growth medium, after 24-hour period of starvation in saline solution. A and B: bacterial protein, C: pellet volume. Curves A and C are means of 3 experiments and pertain to bacteria with low initial ion concentrations (3.0 M.K, 1.14 M.Na, 2.48 Cl, all per kg. cell water). Curve B is the mean of 2 experiments and pertains to bacteria with higher initial ion concentrations (3.85 M.K, 1.75 M.Na, all per kg. cell water). Temperature of incubation: 37°C. pH of incubation medium maintained at 7.0.

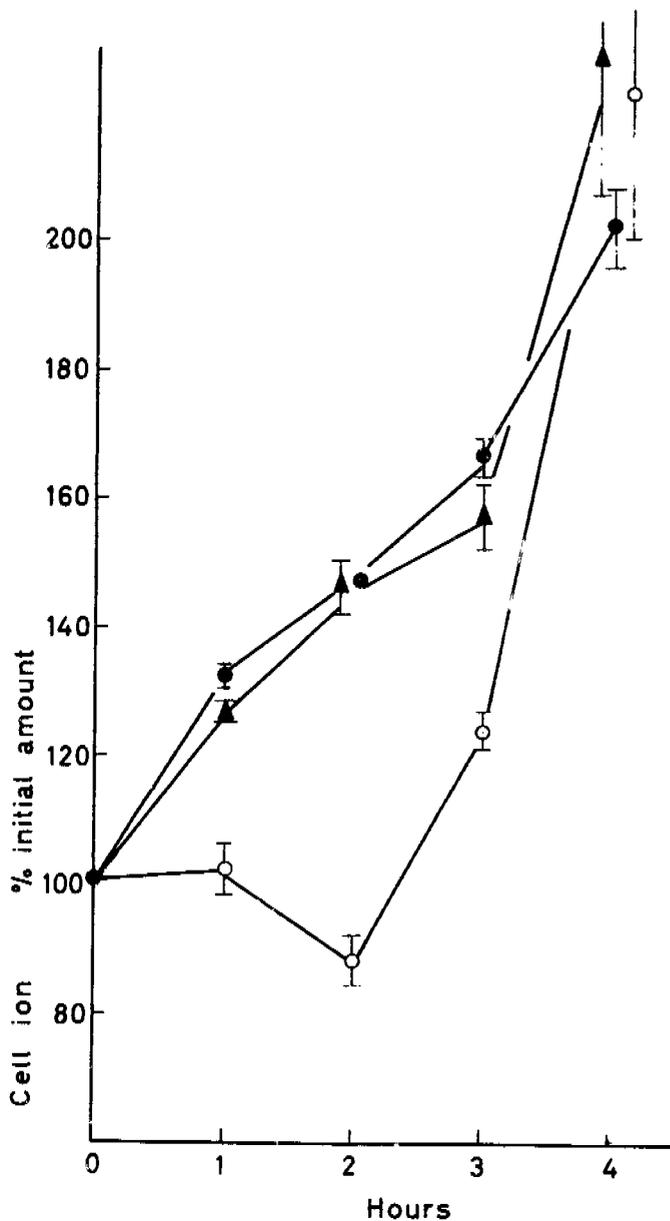


Figure 12.

Increase in ion content in Halobacterium sp. cultures resuspended in growth medium after 24-hour period of starvation in saline solution. Temperature of incubation: 37°C. pH of medium: 7.0. Initial Na and Cl concentrations were low (1.30 M.Na, 2.56 M.Cl, both per kg. cell water). K concentration: 2.76 or 3.85 Moles per kg. cell water. ● :K; ○ :Na; Δ Cl

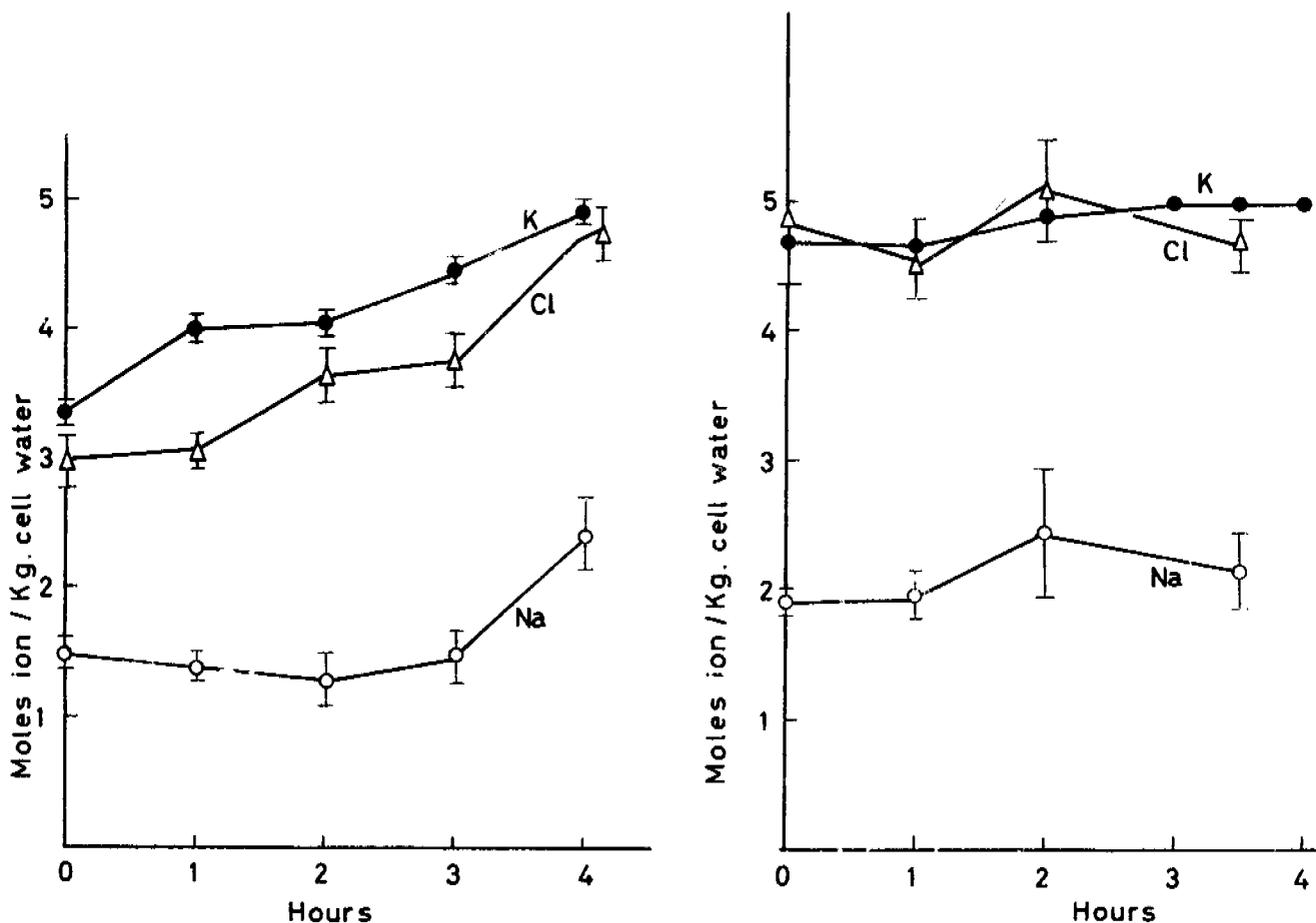


Figure 13

Change in ion concentration in Halobacterium sp. cultures resuspended in growth medium after 24-hour period of starvation in saline solution. Temperature of incubation:  $37^{\circ}\text{C}$ . pH of incubation medium maintained at 7.0. Ion concentrations at end of starvation period, before resuspension in growth medium were:  
 A: 3.0 M.K, 1.14 M.Na, 2.48 M.Cl, all per kg. cell water;  
 B: 3.85 M.K, 1.3 M.Na, both per kg. cell water.

● : K,    ○ : Na,    △ : Cl.

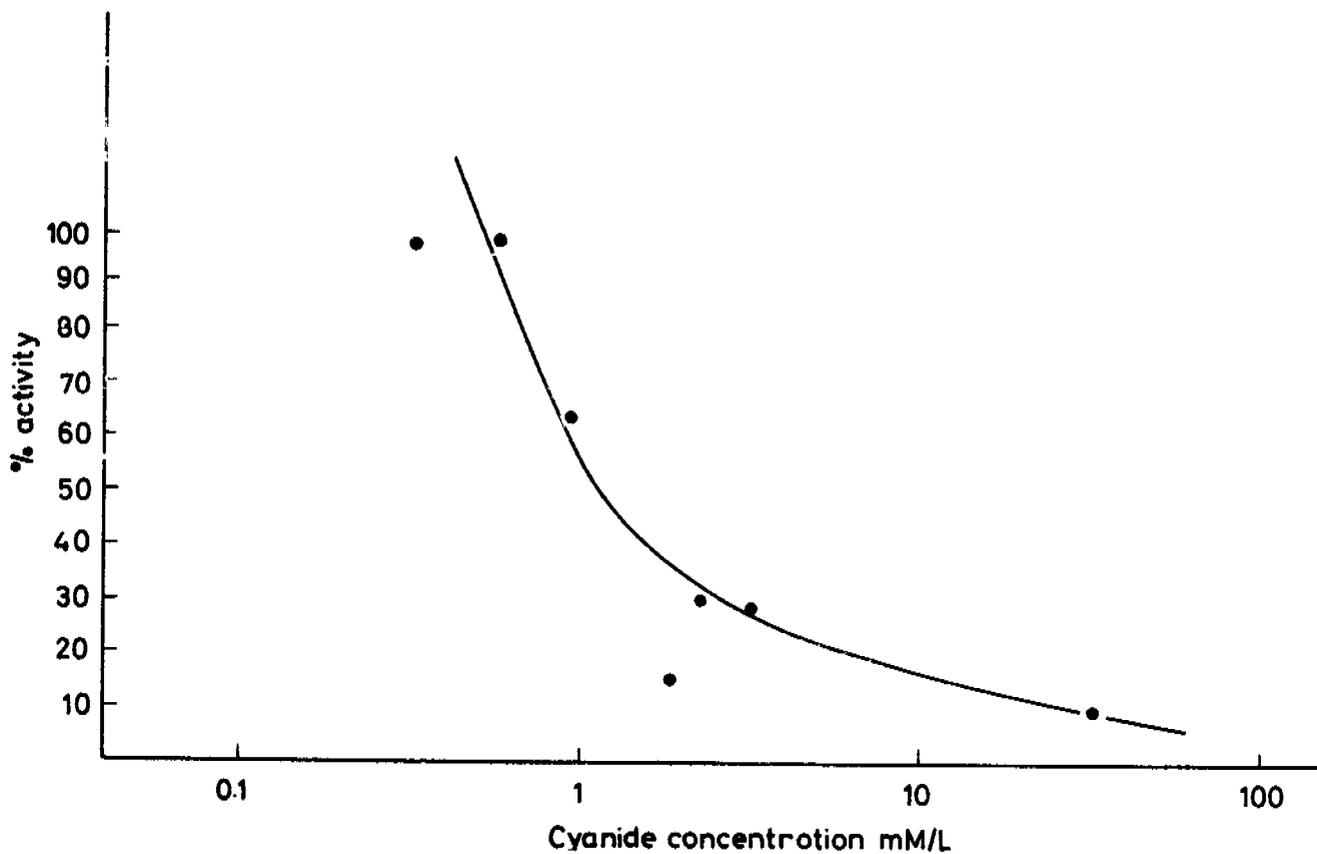


Figure 14

Effect of NaCN concentration on % oxygen uptake of Helobacterium sp. cultures incubated at 37°C at pH 7.0.

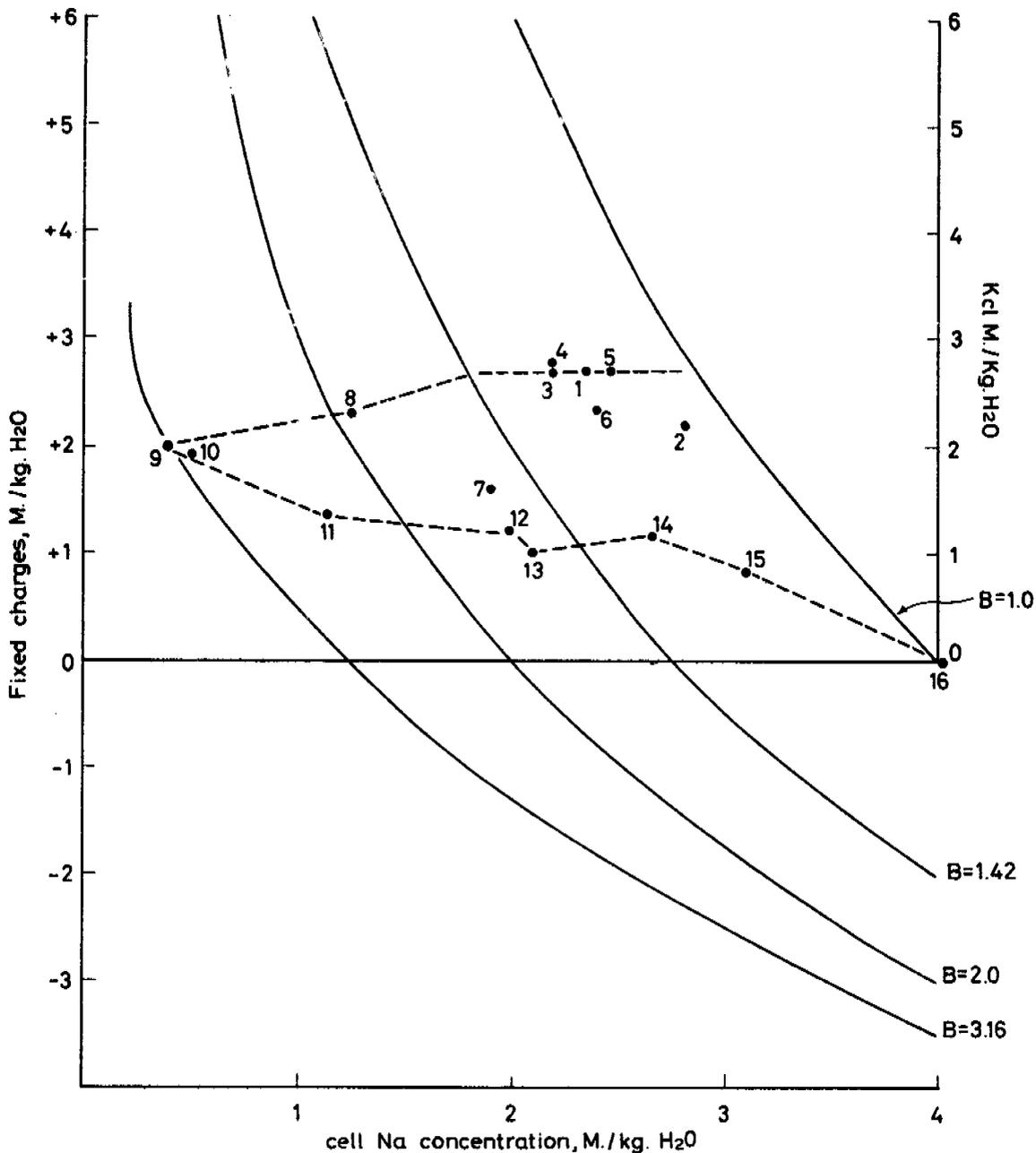


Figure 15

Effect of positive fixed charge concentration within cells on cell Na concentration, calculated according to the Donnan equilibrium.  $B$  = ratio of mean ion activity coefficients inside and outside the cells. Outside NaCl concentration: 3.9 Molal. Solid lines: calculated values. Figures adjacent to experimental points refer to Table 3. Broken line drawn by joining the peripheral points.

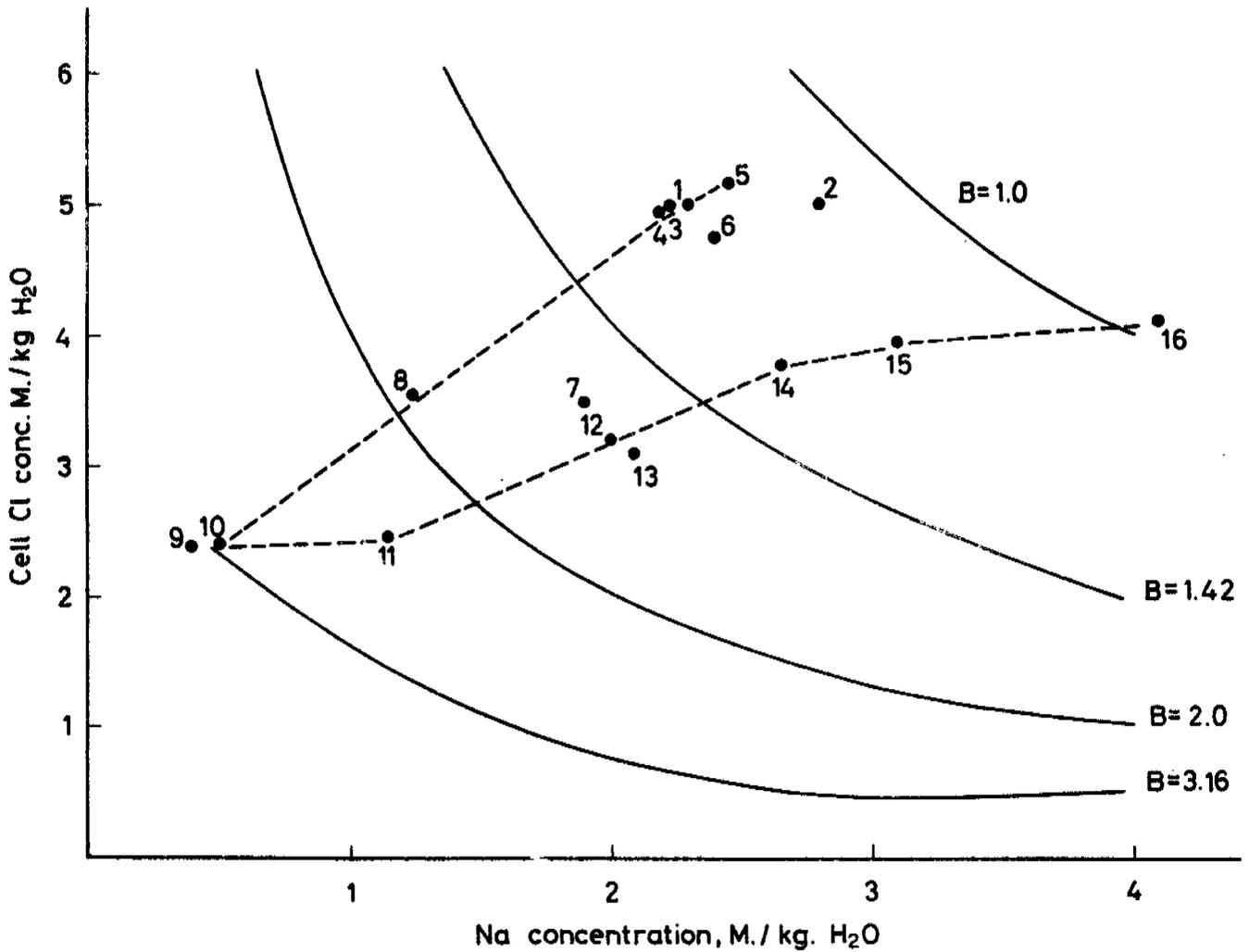


Figure 16

Variations of cell Cl and Na concentrations with different B. Refer to legend of figure 10.

Table 4

Effect of incubation at 2°C for 24 hours on Halobacterium sp.

A. The cultures were stirred vigorously; the medium was not buffered. Mean of 5 experiments  $\pm$  S.E. B. Cultures stirred only for first 3 hours. pH of medium: 7.0.

---

	A.	B.
	<u>Change %</u>	<u>Change %</u>
Cell K content	+144 $\pm$ 2	104 $\pm$ 4
Cell volume	+116 $\pm$ 1	100 $\pm$ 1
Cell protein	100 $\pm$ 2	97 $\pm$ 2

Table 5

Ratio of K:protein in Halobacterium sp. cultures.

Experimental conditions	Ion concentration Moles/kg. cell H <sub>2</sub> O			K:protein μEq/100μg	Number of determinations
	K	Na	Cl		
After 24 hrs. in saline*	3.0±0.05	1.14±0.1	2.48±0.06	1.03±0.04	5
4 addnl. hours in medium	4.9±0.1	2.4 ±0.25	4.75±0.2	1.46±0.1	4
Maximum ion concentrations (Fig. 8B)	5.0	2.2 ±0.3	4.7 ±0.2	1.63±0.1	4

\* Initial optical density of culture: 0.20

Table 6

Ion concentrations in *Halobacterium* under  
different experimental conditions

Conditions	Concentration Moles/kg. cell H <sub>2</sub> O				
	K	Na	Cl	Anion defi- ciency	K <sub>Cl</sub> : Cl-Na
A. Actively-growing bacteria; pH 7.0					
1) Control (fig. 1)	4.75	2.34	5.0	2.09	2.66
2) Initial point (fig. 2)	4.7	2.8	5.0	2.5	2.2
3) " " ( " 4)	3.95	2.2	4.95	1.2	2.7
4) " " ( " 5)	4.95	2.2	4.95	2.2	2.75
5) After resuspension (fig. 8A)	4.9	2.4	4.75	2.65	2.35
6) " " ( " 8B)	5.0	2.45	5.15	2.30	2.70
7) <sup>a</sup> Culture at 0.0.0.25	3.75	1.9	3.5	2.15	1.6
B. Non-metabolising bacteria; no lag before resumption of growth					
8) 24 hours, 0°C.	4.45	1.25	3.55	2.0	1.9
<sup>c</sup> After 24 hours in saline	3.85	1.3	not mea- sured	-	-
C. Non-metabolising bacteria; lag before resumption of growth					
9) 4 hours, argon, 0°C	3.35	0.4	2.4	1.3	2.0
10) <sup>b</sup> Stationary state	3.9	0.5	2.4	2.0	1.9
11) <sup>d</sup> 24 hours, saline	3.0	1.14	2.48	1.66	1.34
12) 3 hours, 1mM NaCN	4.0	2.0	3.2	2.8	1.2
13) 4 hours, argon, 37°C	3.1	2.1	3.1	2.1	1.0
14) <sup>a</sup> At pH 5.0	3.1	2.65	3.78	1.87	1.13
15) <sup>a</sup> At pH 5.5	3.7	3.1	3.95	2.85	0.85
16) <sup>a</sup> At pH 7.8	2.3	4.1	4.1	2.3	0

<sup>a</sup>See ref. (6).      <sup>b</sup>See ref. (10).      <sup>c</sup>Bacteria resuspended from  
cultures at 0.D. 0.06.      <sup>d</sup>Bacteria resuspended from cultures at  
0.D. 0.20.

Table 7

Effect of inhibitors on cell K

% of initial amount

Loss in cell K per  
hour at 37°C

6.8 ± 0.3 (S.E.)

8.7 ± 0.3

Effect of pH of medium on ion concentrations  
of Halobacterium sp.

## INTRODUCTION

During the course of a routine examination of the effects of various external parameters on a Halobacterium species, it was found that pH had large and unexpected effects. The subject was therefore examined in greater detail.

In cultures of the species of Halobacterium under study, cell potassium has been found to be present at concentrations of 3-5 Moles per kg. cell water, the exact concentration depending on the treatment to which the cultures were subjected (1). Reasons have been given for suggesting that K is specifically bound within the cell in 2 forms, a cationic complex balanced by Cl ions ( $K_{Cl}$ ) and an electrically neutral complex balanced internally by an organic anion ( $K_A$ ). It has been shown that the Na and Cl concentrations occurring in this organism can be explained by postulating that positive fixed charges within the cell (equivalent to  $K_{Cl}$ ) exert a Gibbs-Donnan effect by means of which the Na and Cl concentrations are kept at equilibrium values. The bacteria have been shown to be extremely permeable to large organic molecules (3). Changes in concentration of Na and Cl are brought about by a combination of changes in the concentration of the positive fixed charge and of the mean ion activity coefficient.

This paper demonstrates the high permeability of this Halobacterium species to the  $H^+$  and small inorganic ions. It also indicates the occurrence of conformational changes and reinforces the conclusion that the Halobacterium cell is an open system maintained at equilibrium with its surroundings by means of changes within the cell proteins.

## M E T H O D S

Methods of culture of the Halobacterium sp. used have been described elsewhere (2), together with methods of analysis and calculation.

### Incubation of bacterial cultures at a given pH value.

Cultures in the logarithmic phase at an optical density of 0.25 were used. The method of incubation has been described (1). The pH was fixed at the given desired value by addition of small volumes of NaOH or HCl diluted to a final concentration of 0.1 N by addition of growth medium containing 3.9 Molal NaCl. For a complete description of the growth medium, see Table 8 in reference (2). The bacteria responded to changes in pH of the medium by vigorous acidification or alkalinisation of the medium; the pH of the culture was maintained at the desired value by further small additions of 0.1 N NaOH or HCl.

## R E S U L T S

Of all the environmental factors studied so far, the pH of the medium has been found to have the profoundest effect on cell volume and on cell ion content. The range of pH studied was from 4.0 to 8.0.

It was most unexpected to find that the cell volume per unit of protein changed as the pH of the medium changed. These fluctuations in volume are plotted in figure 17 taking the volume at pH 7 as 100. The time of incubation at any given pH value was 1 hour if the change in pH was of 1 pH unit or less, and 2 hours if the change was above 1 pH unit. The pH range 7.0-7.8 was the only one

at which no volume changes occurred. At pH 7.8 the cells swelled to 166% of their previous volume. The changes occurring at pH 7.8 will be dealt with in greater detail in a later section.

From pH to 5.5 there was a progressive shrinkage, so that at pH 5.5 the cells were approximately half the size that they were at pH 7. Below pH 5.5 the cells swelled until they almost regained the volume they had at pH 7.

When the amount of K per cell was measured, it was found that the largest amounts of K, per unit of protein, were found at pH 7. Between pH 7 and 8 there were no changes in amount of cell K. Below pH 7 cell K, in amount of K per unit of protein, fell until at pH 4 no cell K was left at all. The fall in cell K occurred in a step-wise manner; the largest increments occurred from pH 7.0-6.6 during which 24% of the total K was lost, pH 6.0-5.5 (12% loss) and pH 5.0-4.0 (59% loss).

Since the changes in cell volume and in amount of cell K ran roughly parallel between pH 7.0 and 5.5 the concentration of cell K remained steady in this range (see figure 18). No change in cell dry weight was found within this pH range. At pH 7.8 there was a fall in cell K concentration, due entirely to cell swelling. Below pH 5.5 cell K concentration fell, partly because of loss of cell K and partly because of cell swelling.

Cell Na concentration remained well below the level of the outside concentration except at pH 7.8 where it was not significantly different from the outside concentration. In the pH range of 4.5-7.5 the Na concentrations made a rough mirror-image of the

changes in pellet volume. Thus the maximum cell Na concentration was at pH 5.5, which was also the pH of minimum pellet volume.

It was not possible to determine cell Na concentration at pH 4.0 because of clumping of the bacteria during centrifugation at this pH. There were indications, however, that the cell Na content was very high.

From pH 5.5 to 7.5 the cell Cl concentration was  $3.45 \pm 0.1$  Moles per kg. cell water. At pH 7.8 the cell Cl concentration of 4.15 Moles per kg. cell water, differed little (probably not significantly) either from the outside concentration or from the cell Na concentration.

At no pH, was there enough Cl to balance the sum of the cations (see Table 6 in ref. 1). The deficit in anion content varied from 1.6 to 2.3 Moles per kg. cell water and implies that the balance of the negative charges must have been supplied by the cell, in the form of organic anions, throughout the pH range studied.

These observations agree with a number of analyses of protein of the halobacteria, all of which have concluded that the proteins from these bacteria are more acidic than are those from other organisms (4).

In the range pH 5.0 to 7.8 all of the changes described were completely reversible. The sequence of events which occurred when the pH of the medium was increased from 7.0 to 7.8 are described in figure 19.

Let us first consider cell volume, reflected by changes in pellet volume. After 2 minutes at pH 7.8 the pellet had swollen to  $1.4 \text{ mm}^3$  per ml. suspension (original volume at pH 7.0:  $1.0 \text{ mm}^3$  per ml. suspension) i.e. a swelling of 40%. After 30 minutes at pH 7.8 the pellets reached their maximum size and had swollen by 66% of the original volume. Thereafter, there was a slow reversal, and the bacteria shrank once more. Possible reasons for the shrinkage will be discussed later.

The amount of cell K remained constant during the entire period that the bacteria were at pH 7.8. The K concentration, however, fell from its initial value of 3.7 Moles per kg. cell water to 2.35 Moles per kg. cell water; this latter concentration was reached 30 minutes after the pH had been increased to 7.8. As the cells shrank, the cell K concentration rose.

The amount of cell Na rose very abruptly when the pH of the medium increased to 7.8. Thirty minutes after the pH increase cell Na rose to 3 times the original amount but owing to the cell swelling, the Na concentration no more than doubled, and in fact at this time was not significantly different from the outside Na concentration ( $\text{Na}_{\text{in}} : 4.15 \pm 0.2$  Moles per kg. cell  $\text{H}_2\text{O}$ ;  $\text{Na}_{\text{out}} : 3.9$  Molal). As time proceeded, the amount of cell Na decreased and the concentration fell below the medium Na concentration at the time of the final reading ( $\text{Na}_{\text{in}} : 3.15 \pm 0.3$  Moles per kg. cell water;  $\text{Na}_{\text{out}} : 3.9$  Molal).

Cell Cl changes followed the changes in cell Na, with one difference: less Cl was gained during the initial swelling period and less was lost during the second period of shrinkage.

The bacteria acidified the medium throughout the period that they were at pH 7.8. The acidification was countered by addition of N/10 NaOH, so as to maintain the pH at 7.8.

When the pH of the medium was kept at 7.8 for only 2 minutes and then returned to 7.0 the cell swelling and Na intake which occurred at pH 7.8, were largely reversed as the pH was lowered (see Table 8), or at any rate within the time needed to take the measurement, say 2-3 minutes. Thus the bacteria responded very quickly to the changes in pH, implying that the cell membrane is highly permeable to pH.

In a similar type of experiment, bacteria pre-incubated in saline solution for 24 hours were exposed to pH 7.8. The saline solution consisted of 3.9 Molal NaCl, 150mM MgSO<sub>4</sub>, 1.4 mM CaCl<sub>2</sub>, 2.5.10<sup>-4</sup> mM MnCl<sub>2</sub>. These bacteria had been totally deprived of any C or N substrate during their incubation in saline, and were apparently metabolically inactive: they did not absorb O<sub>2</sub> nor bring about any pH-change in the medium, nor synthesize protein. Such bacteria were totally unaffected when the pH of the medium was increased to 7.8; in 5 experiments no change in the concentration of any ion was detected (see Table 9).

Halobacterium can apparently withstand pH values above 8, since bacteria grew normally after being incubated at pH 9 for 1 hour after which the pH of the medium was returned to 7. The effects, if any, of pH values above 8 could not be studied since they bring about the precipitation of a white flocculent mass of magnesium phosphate from the medium.

Figure 20 shows the sequence of events that occurred when bacteria, originally at pH 7 were exposed to pH 6.0 for 60 minutes, after which the pH was returned to 7.0. During the initial hour at pH 7 the bacteria grew steadily, as is revealed by the increases shown in all of the parameters measured. When the pH was lowered to 6.0 there were decreases in amount of cell K and in pellet volume. The loss in cell K is shown to be completed in 40 minutes. Cell Na and Cl remained steady during this time. Since no net change in Na or Cl occurred to balance the loss of K, it would appear that K was lost in exchange for  $H^+$  entering the cells.

After the pH was returned to 7.0, all of the K which had been previously lost, was regained within 20 minutes. During this period there was a burst of acid secretion which continued for as long as the cells were regaining K. Later, the bacteria alkalinised the medium in the usual way. Gains in cell Cl and Na occurred during the first hour after which the pH was restored to 7.0. An exchange of K for Na is thus ruled out. The big increase in Cl that occurred during recovery indicates that about 70% of the K entering the cells must have been balanced by Cl. The remaining 30% of the K may have been exchanged for  $H^+$ .

#### DISCUSSION

The results reported above have made it clear that the cell ions of the Halobacterium species studied have been greatly affected by the pH of the medium. The previous paper in this series (1) showed that the maintenance of ion gradients across the bacterial membrane was not effected by the continuous expenditure of

metabolic energy. It seems clear, therefore, that the pH did not act by influencing rate of metabolism, but that its effects are more probably due to change in the conformation of the cellular macromolecules. Volume changes can be demonstrated externally owing to the lack of a rigid cell-wall (5) which permits the bacterial cell to change in volume.

Let us first consider the changes in cell volume which occurred after changes in the pH of the medium. The smallest bacterial volume per unit of protein was found at pH 5.5. The cell volume increased as the pH was either raised to 7.0 or lowered to pH 4.0. In these respects the bacteria behaved like a flexible, ampholytic protein gel with an iso-electric point at pH 5.5 (6). It is tempting to suggest that the charges in the pH 4.0-5.5 range are due to the carboxyl side-chains of the bacterial proteins (7). Changes in the pH 6.0-7.0 range may be due to the loss of charge of groups more basic than the carboxyl radicle; the imidazole group was found to have its pK in this region, when present as a component of several proteins which have been studied (7), but is unlikely to be responsible for the effects observed, owing to the small amount of histidine found on analysis (4).

Between pH 7.0 and 7.8 no change in volume was observed. Another change took place, this time very sharply, at pH 7.8 at which point the cell volume swelled to 1.66 times its value at pH 7.0, perhaps because of loss of charge of the  $\alpha$ -amino groups present as side-chains in proteins. According to Cohn and Edsall, the pK of these groups is 7.6-8.4 (7).

It is concluded that the bacteria tended to swell at the extremes of the pH range studied. In these regions the charge-densities on the proteins were probably the highest.

Bacteria incubated in saline did not swell at pH 7.8. Although the reason for this is not clear, it may once more indicate the importance of protein conformation in determining response to pH; it was shown earlier (1) that the mean ion activity coefficient ratio (B) was higher in starved bacteria than in those supplied with nutrient. This was explained as caused by a change in protein conformation.

The extraordinary rapidity of some of the volume changes observed (see for example Table 8) demonstrate the high permeability of the bacteria to  $H^+$ , water and ions and must have been facilitated by the lack of any rigid cell-wall. It appeared, however, that the bacteria may possess some ability to control their internal pH: this was demonstrated in figure 19, when the cell swelling caused by the increase in pH to 7.8 was partially reversed during the course of an hourly period of incubation. During this time it was observed that the bacteria tended to acidify the medium and it is probable that the pH of the bacterial cytoplasm fell below the outside value.

The amount of cell K was found to be completely pH-dependent. Bacteria incubated at pH 4 were completely devoid of K; the amount of cell K increased with pH until the maximum amount per unit of protein was found at pH 7.0. In contrast, the concentration of cell K rose as the pH of the medium was increased from 4 to 5.5, due both to cell shrinkage and to increase in amount of cell K. From pH 5.5

to 7.0 the cell K concentration did not change since the increase in amount of cell K was kept pace by the increase in cell volume (% cell water remaining constant). At pH 7.8 there was a second fall in K concentration, due entirely to cell-swelling.

The nature of the K-complex - whether  $K_{Cl}$  or  $K_A$  - was affected by the pH of the medium. The lowest concentrations of  $K_{Cl}$  occurred at the extremes of the pH range (4.5-5.5 and 7.8) and even approached zero at pH 7.8. The concentration of  $K_{Cl}$  and  $K_A$  at different pH values are listed in Table 9 (1).

It is probable that the pH of the medium acts on the amount of cell K by affecting the stability of the postulated K-complex. Such an effect has been seen in nigericin (8). The K concentration is determined by interactions between the postulated pH effects on the stability of the K-complex and on the volume, or conformation, of the cell protein. These two effects are also responsible for the low concentration of  $K_{Cl}$  at the lower pH range. The reason for the shift of  $K_{Cl}$  to  $K_A$  at pH 7.8 is not clear.

On considering Na and Cl concentrations, it was found that the concentration of Na varied between 1.9 and 3.0 Moles per kg. cell water from pH 4.5 to 7.5. The Cl and Na concentrations generally ran in parallel. However, at pH 7.8 the Na and Cl concentrations were at least as high as in the bathing medium. This was the only case, in the entire course of this work, in which the Na concentrations were equalised across the membrane. All of the Na and Cl concentrations are explainable in terms of Gibbs-Donnan equilibria (see Table 6 and figures 15 and 16 in ref. 1).

The equalization of Na concentrations across the cell membrane which occurred at pH 7.8 is to be expected in the absence of any positive fixed charges provided by  $K_{Cl}$ , the concentration of which dropped to nearly zero at this pH. After a period of 1 hour at pH 7.8, it was observed that the Na concentration fell significantly below the outside level; this is explainable by postulating a fall in pH in the bacterial cytoplasm, just as was done to explain the cell shrinkage which occurred at this time.

If the cells do indeed behave like a Gibbs-Donnan system, then the  $Na^+$  and the  $H^+$  must be distributed among the cell and the outside medium in like fashion. Thus the pH inside the cell must be lower by 0.2 to 0.5 pH unit than the pH of the outside medium, except at pH 7.8 when equalization must occur.

It is concluded that there were no direct effects of pH on cell Na or Cl concentrations, but that all the changes observed were mediated via direct effects on protein conformation and on cell K. It has been shown that the cell behaves like a flexible, ampholytic protein gel, the changes in volume of which are demonstrable because of the lack of a rigid cell-wall. The pH-dependency in amount of cell K has been ascribed to an instability of the K-complex at low pH.

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## LEGENDS

### Figure 17

Effect on cell K and volume of incubation in media at different pH values. Results are expressed as % of the values at pH 7 per unit of bacterial protein. Temperature of incubation: 37°C. Cultures were incubated until equilibrium was attained. Each point is the average  $\pm$  S.E. of not less than 4 experiments.

### Figure 18

Cell ion concentrations of Halobacterium sp. after incubation in media at different pH values. For conditions of experiments see legend to figure 17. The values at pH 7.8 are those measured after a 30-minute incubation period. ● : K, ○ : Na, Δ : Cl.

### Figure 19

Effect of incubation at pH 7.8 on cell ion contents, protein and volume of Halobacterium sp. Temperature of incubation: 37°C. Mean  $\pm$  S.E. of 4 experiments. ● : K, ○ : Na, Δ : Cl.

### Figure 20

Effect of incubation at pH 6.0 on cell ion contents, volume and protein of Halobacterium sp. Temperature of incubation: 37°C. Mean  $\pm$  S.E. of 2 experiments.

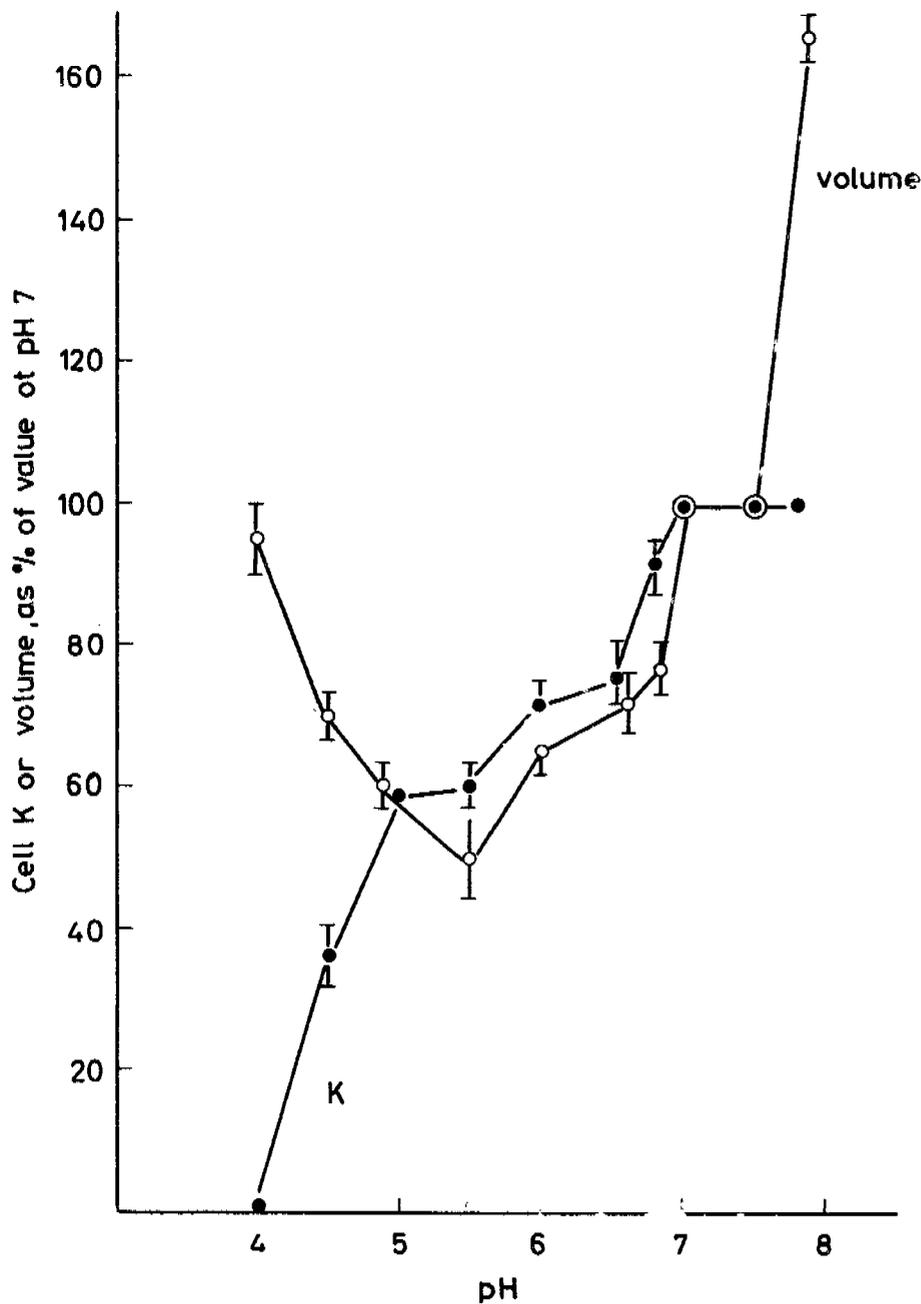


Figure 17

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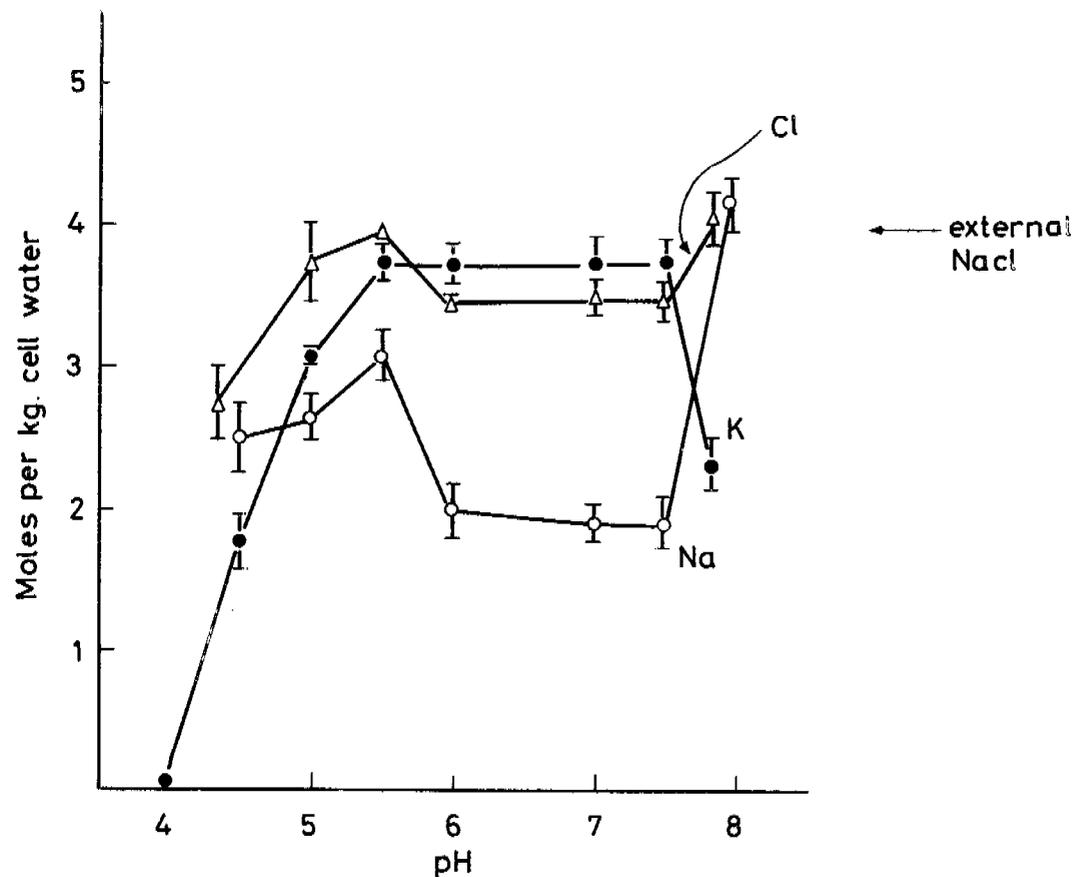


Figure 18

Cell ion concentrations of Halobacterium sp. after incubation in media at different pH values. For conditions of experiments see legend to figure 1. The values at pH 7.8 are those measured after a 30-minute incubation period. ● : K, ○ : Na, Δ : Cl.

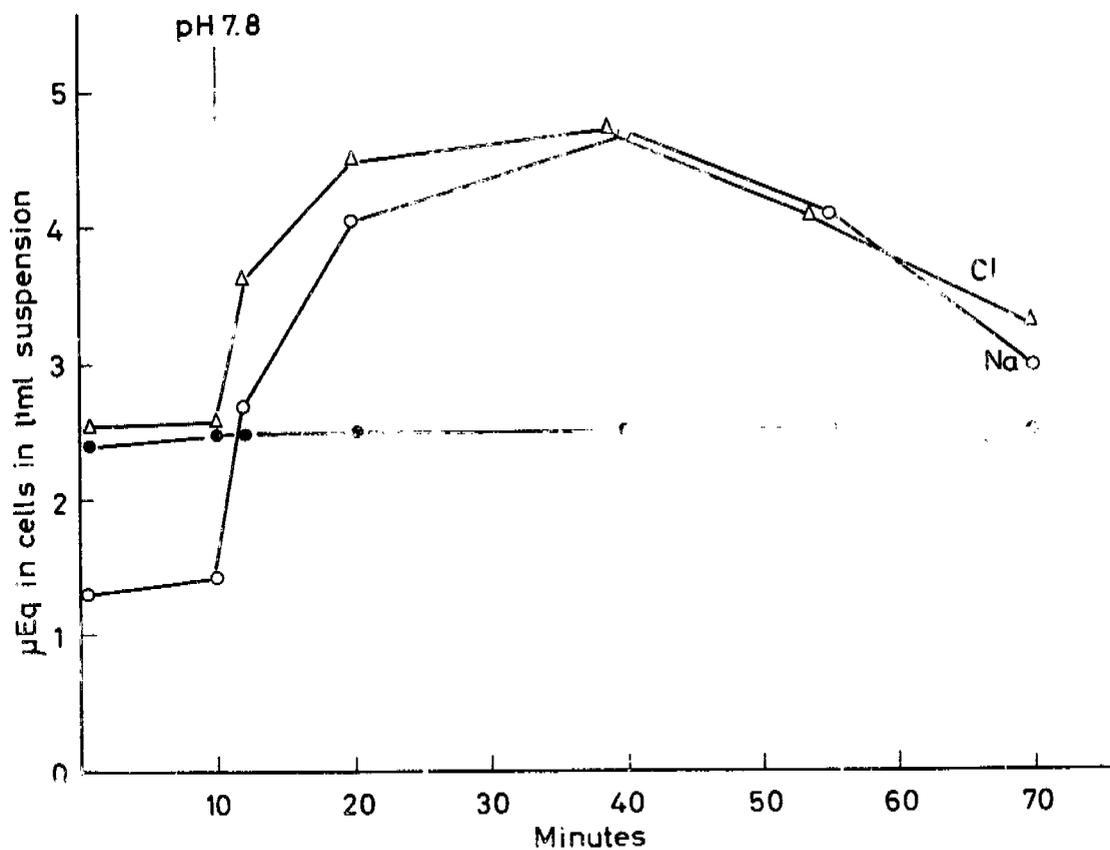
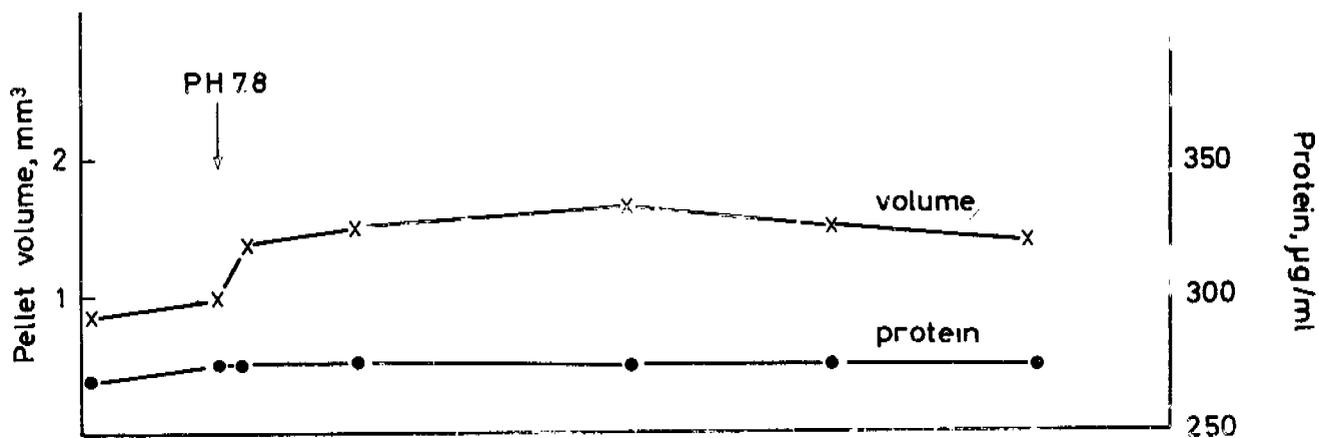


Figure 19

Effect of incubation at pH 7.8 on cell ion contents, protein and volume of Halobacterium sp. Temperature of incubation: 37°C. Mean  $\pm$  S.E. of 4 experiments. ● : K, ○ : Na, Δ : Cl.

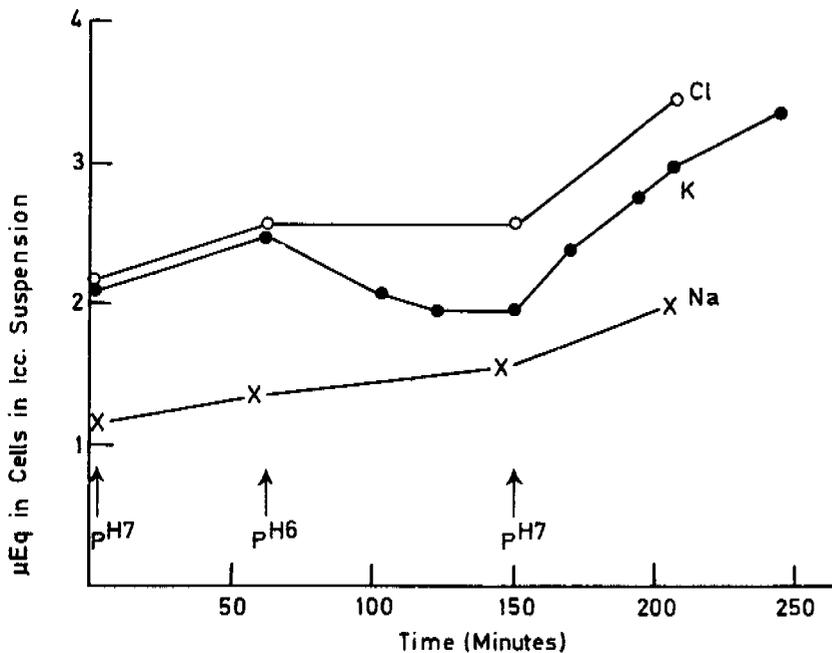
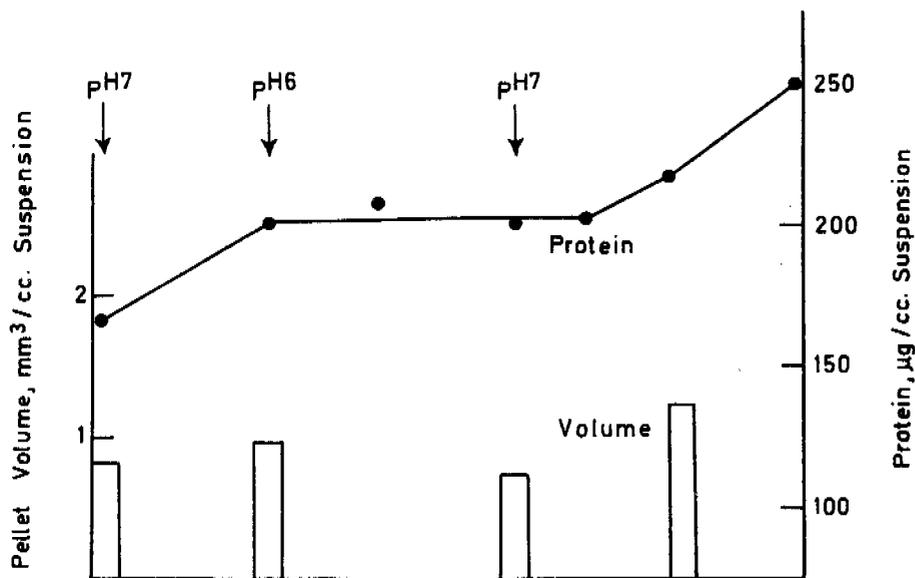


Figure 20

Effect of incubation at pH 6.0 on cell ion contents, volume and protein of Halobacterium sp. Temperature of incubation: 37°C. Mean  $\pm$  S.E. of 2 experiments.

Table 8

	<u>As % of initial conditions</u>		
	<u>Volume</u>	<u>uEq in cells/ml. suspension</u>	
		<u>Na</u>	<u>Cl</u>
Initial conditions, pH 7.0	100	100	100
After 2 mins. at pH 7.8	140	286 ± 26	128 ± 5
After 2 further mins. at pH 7.0	113	157 ± 20	92 ± 4

(Mean of 5 experiments)

Table 9

Effect of incubation at pH 7.8 on Halobacterium pre-  
incubated in saline solution for 24 hours.

Mean of 5 experiments ± S.E.

	<u>Moles / kg. cell H<sub>2</sub>O</u>		
	<u>K</u>	<u>Na</u>	<u>Cl</u>
Initial (pH 7.0)	3.0 ± 0.05	1.14 ± 0.1	2.48 ± 0.06
After 30 mins. at pH 7.8	3.0 ± 0.05	1.14 ± 0.1	2.48 ± 0.06

Light induced proton uptake in  
Dunaliella parva.

ABSTRACT

Light-induced  $H^+$  uptake ("proton pump") has been observed with whole cells of the halophilic alga Dunaliella parva. The rate of  $H^+$  uptake is in the range of 2-6 uequiv  $H^+$ /min per mg chlorophyll. The extent of the reaction is about 9-10 uequiv  $H^+$ /mg chlorophyll at an initial pH of 6.2. The extent increases linearly with increase of initial pH. The rate of  $O_2$  evolution decreases parallel to the decrease in rate of  $H^+$  uptake. Tris and other buffers prevent the decrease in rate of  $O_2$  evolution, and increase the extent of  $H^+$  uptake. (3,4-Dichlorophenyl)-1,1-dimethylurea inhibits  $H^+$  uptake, but does not affect the decay in the dark. Atebrin and, to a lesser extent,  $NH_4Cl$  increase the rate of decay. Carbonyl cyanide m-chlorophenylhydrazone does not affect the rate of decay at all.

Ben-Amotz, A. and Ginzburg, B.Z. 1969. Light-induced proton uptake in whole cells of Dunaliella parva. Biochim. Biophys. Acta, 178:144-154.

## CONCLUSIONS

Work has been performed on bacteria belonging to a species of the genus Halobacterium and on green unicellular algae belonging to the species Dunaliella parva. Both organisms are characterised by their requirement for a high salt concentration for growth and the maintenance of life; Halobacterium sp. grows in a medium containing 3.5 Molar NaCl, and D. parva in 1.0-1.5 Molar NaCl.

Both organisms have very permeable membranes; the Halobacterium membrane is permeable to molecules of up to 40,000 in molecular weight (e.g. polyvinylpyrrolidone). A region of the D. parva cell is permeable to sucrose and inulin (M.W. 6000). Thus the organisms have the most permeable membranes known up to now.

The concentrations of K, Na, Cl were measured throughout the life-cycle of cultures of the Halobacterium species. Bacteria in the growth phase were characterised by the following concentrations:- K:- 4-5 Moles, Na:- 1.5-2.5 Moles, Cl:- 3.5-5.0 Moles, all per kg. cell water. Bacteria in the stationary phase were characterised by the following:- K:- 3.9 Moles, Na:- 0.5 Moles, Cl:- 2.4 Moles, all per kg. cell water. The ion concentrations in the medium are 3.9 Molal NaCl and 5mM KCl. It follows that some of the cell ions must be bound, for the following reasons:-

- a) It is impossible to dissolve in water at ordinary temperatures and pressures 4M KCl and 2M NaCl.
- b) Since the membrane is very leaky, high concentrations of KCl could not be retained within the cell if the KCl were all in solution.

c) Since Halobacterium cells are known to be naked, and do not possess a rigid cell-wall, high concentrations of salts within the cell would cause an explosion. It follows that a portion of the salts cannot be in solution, so that there is no hydrostatic pressure difference across the cell membrane.

Later work showed that it is probably  $K^+$  which is bound within the cell: high K-gradients could be maintained across the membrane in absence of metabolic energy output; also, K disappeared in a slow, linear manner from cells incubated in argon, whereas the disappearance should have been quick and logarithmic, had the K been in solution within the very permeable cells. The K is postulated to form 2 complexes within the cell. In the first, the K retains its charge which is balanced by cell Cl ( $K_{Cl}$ ). In the second, the K charge is balanced internally ( $K_A$ ). Examples of such complexes have been discovered recently, and are given by valinomycin and nigericin.

Non-metabolising bacteria were characterised by low Na and Cl concentrations (i.e. a high Na gradient across the cell membrane). It was found that Na and Cl were lost in equimolecular amounts with little change in cell K, on cooling the bacteria, or in stationary state bacteria. These results can be explained by postulating that there is a Gibbs-Donnan equilibrium across the cell membrane, and that changes in the concentration of fixed positive charges within the cell ( $K_{Cl}$ ) and/or changes in the mean ion

activity coefficient bring about changes in the cell Na and Cl . Changes within the cells are facilitated by the very permeable cell membrane.

Changes in the pH of the ambient medium were found to affect the amount of cell K and the cell volume. These latter could be demonstrated owing to the absence of a rigid cell-wall. The quickness of the reactions observed emphasized the high permeability of the membrane to H<sup>+</sup>, small ions and to water.

It was concluded that the Halobacterium cell behaves as a flexible, ampholytic, protein gel maintained at equilibrium with the ambient medium by means of conformational changes within the cell proteins. In contrast, the ordinary, mesophilic cell is relatively rigid and cell ions are maintained at concentrations far from equilibrium by means of a continuous expenditure of metabolic energy.

The high permeability of D. parva allowed proton-uptake in illuminated cells to be studied in far better conditions than in any cell-system up to now. The H<sup>+</sup> gradients built up were found to be intermediate in the synthesis of high-energy phosphate compounds.